Positive Allosteric Modulation of Inhibitory Neurotransmitter Receptors - Novel Mechanisms and Targets to Restore Spinal Inhibition in Persistent Pain

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Mario Andrés Acuña Miranda

aus

Chile

Promotionskommission

Prof. Dr. Hanns Ulrich Zeilhofer (Leitung der Dissertation)
Prof. Dr. Raimund Dutzler
PD Dr. Dietmar Benke

Zürich, 2016

Contents

Summary

Evolution has provided us with the possibility to avoid potentially damaging environmental impacts. Those eventual or actual damaging events are sensed by specialized sensory neurons called nociceptors. Nociceptive signals generated in the peripheral tissue travel from there to the first relay center in the central nervous system, the spinal cord. Here, the information is decoded, filtered or amplified, and eventually conveyed to the brain, where the conscious sensation of pain is formed. Malfunctioning of this nociceptive system can lead to persistent pain without apparent benefit. Diminished inhibitory neurotransmission in the superficial spinal dorsal horn is a pivotal event in the generation and maintenance of pathological pain syndromes. Recent studies have shown that spinal disinhibition can arise from decreased glycinergic and γ-aminobutyric acid (GABA)ergic neurotransmission. Accordingly, pharmacological enhancement of glycinergic or GABAergic synaptic inhibition in the spinal dorsal horn should be well-suited to restore proper inhibition and eventually have a beneficial effect on chronic pain syndromes. However, in order to avoid side effects originating from supraspinal sites, strategies need to be developed that restrict these interventions to the spinal cord. Targeting glycine receptor or $GABA_A$ receptor (GlyRs or $GABA_A$Rs) subtypes that are specifically expressed or enriched in the spinal cord may provide such opportunities.

In the first part of this thesis, I investigated the modulation of GlyRs by the non-anaesthetic propofol derivative 2,6-di-tert-butylphenol (2,6-DTBP). I found that 2,6-DTBP reverses inflammation-mediated disinhibition through a specific interaction with heteromeric phosphorylated synaptic α3βGlyRs. Using electrophysiological techniques, I demonstrated that recombinant homomeric α3GlyRs are sensitive to the modulation of 2,6-DTBP. However, the inclusion of the β subunit to the receptor complex dampens the sensitivity to 2,6-DTBP. Our molecular modelling suggests that in heteromeric α3βGlyRs this modulatory site is accessible to 2,6-DTBP only after a PKA-dependent phosphorylation. In native mouse spinal cord tissue, 2,6-DTBP modulated synaptic GlyR currents only after priming with PGE_2, consistent with our molecular modelling data. In mouse models of inflammatory pain, 2,6-DTBP reduced inflammatory hyperalgesia in an α3GlyR-dependent manner. These data suggest that an additional level of selectivity can be reached targeting inflammation-primed α3GlyRs, reducing the likelihood of off-target effects.

The second part of my thesis was directed to characterize the modulatory activities of selected (non- or less sedative) benzodiazepine-binding site ligands (BDZs) acting at canonical and non-canonical BDZ-binding site of $GABA_A$Rs. Several lines of evidence

demonstrate the importance to target α2-cointaining $GABA_A$Rs for the analgesic effects of BDZs over α1$GABA_A$Rs, which mediate the sedative effects. I therefore aimed to characterize selected BDZs in order to find compounds with an α2/ α1 selectivity ratio. Canonical BDZ-binding sites are formed by the interface of one out of four α subunits (α1, α2, α3, or α5) and the γ2 subunit. I found that N-desmethyl clobazam (NDMC), the main metabolite of the licensed anti-epileptic drug clobazam (CBZ) possess better selectivity to the antihyperalgesic α2-containing $GABA_A$Rs over the classical BDZ diazepam (DZP). Among the six compounds tested, imidazotriazine TPA023B showed the best α2/α1 ratio, in agreement with previous findings. Non-canonical BDZ-binding sites are made up by 'non-γ2'$GABA_A$Rs. Among the three γ subunits, γ1 is also expressed at potentially relevant levels in the spinal cord, and its localization and expression in the brain is lower compared to γ2. In addition, there is evidence indicating that other factors in addition to the α subunit subtypes contribute to the pharmacodynamic profile of BDZs. I therefore characterized the activities of several non- or less sedative BDZs at γ1-containing $GABA_A$Rs. The benzodiazepine HZ-166 showed a higher efficacy at γ1$GABA_A$Rs than at γ2$GABA_A$Rs. When compared to other BDZs, HZ-166 showed the highest γ1 efficacy at sub-saturating concentrations. HZ-166 compound might therefore be useful to test antihyperalgesic effects of spinal γ1$GABA_A$Rs.

Taken together, this thesis describes a novel mechanism for specific pharmacological targeting of "pain relevant" GlyRs and identified a new tool for the analysis of γ1$GABA_A$Rs in spinal pain control.

Zusammenfassung

Die Evolution hat uns mit einem Mechanismus ausgestattet, der es uns erlaubt potentiell gefährliche und schmerzvolle Umwelteinwirkungen auf den Körper zu vermeiden. Diese Reize werden durch spezialisierte sensorische Neurone, den Nozizeptoren in den peripheren Geweben, wahrgenommen. Von den Nozizeptoren aus wird die Reizinformation an das im Rückenmark gelegene Hinterhorn weitergeleitet, wo sie zum ersten Mal auf eine nachfolgende Nervenzelle umgeschaltet wird. Das Umschalten auf die nächste Nervenzelle dient neben der Weiterleitung zum Hirn als Filter, Entschlüssler and Amplifikator des Reizes. Im Gehirn wird die Sinnesinformation dann schliesslich bewusst wahrgenommen und weiter verarbeitet.

Störungen in diesem System können zu einem anhaltenden Schmerz ohne sichtbaren Nutzen führen. Eine solche Störung ist zum Beispiel eine verminderte neuronale Hemmung in den oberen Schichten des Hinterhorns. Studien haben gezeigt, dass eine Enthemmung durch reduzierte glyzinergische und GABAergische (γ-Aminobuttersäure) Neurotransmission zu einem solchen pathologischen Schmerzempfinden führen kann. Dementsprechend sollte das Gegenteil, die Verstärkung der synaptischen Hemmung durch Glyzin und GABA, mittels pharmakologischer Substanzen zu einer Verminderung des chronischen Schmerzleidens führen. Um Nebenwirkungen supraspinalen Ursprungs zu vermeiden braucht es eine Strategie, um die pharmakologische Intervention im Bereich des Rückenmarks einzuschränken. Eine Möglichkeit dafür, wäre zum Beispiel Glyzin-Rezeptoren (GlyRen) oder $GABA_A$-Rezeptoren ($GABA_A$Ren), welche spezifisch im Rückenmark exprimiert oder angereichert sind, zu modifizieren.

Im ersten Teil der Dissertation habe ich die Modulation der GlyRen durch 2,6-di-tert-butylphenol (2,6-DTBP), einem Propofol Derivat, erforscht. Eine entzündugnsinduzierite Enthemmung im Hinterhorn konnte durch die Interaktion von 2,6-DTBP mit heteromeren und phosphorylierten α3βGlyRen rückgängig gemacht werden. Meine elektrophysiologischen Experimente zeigen, dass rekombinante homomerische α3GlyRen durch 2,6-DTBP moduliert werden können, die Einführung der β-Untereinheit jedoch die Sensitivität zu 2,6-DTBP schwächt. Unser molekularbiologisches Model geht davon aus, dass die Modulationsstelle im heteromeren α3βGlyRen nur dann für 2,6-DTBP zugänglich ist, wenn der Rezeptor durch das PKA Enzym phosphoryliert wurde. Wie durch unser Modell vorhergesagt, konnten im Rückenmark einer nativen Maus die GlyR-Ströme nur durch vorherige Inkubation mit PGE_2 durch 2,6-DTBP moduliert werden. In Mausmodellen für

Entzündungsschmerz konnte 2,6-DTBP durch die Modulation an α3GlyRen den Schmerz mindern. Diese Daten zeigen, dass ein zusätzliches Level an Selektivität gewonnen werden kann, wenn entzündungsaktivierte α3GlyRen für die pharmakologische Modulation angepeilt werden.

Der zweite Teil meiner Dissertation war der Charakterisierung der modulierenden Tätigkeiten von ausgewählten (nicht oder wenig sedativen) Benzodiazepin (BDZ) Bindungsstellen-Liganden gewidmet. Diese wirken auf kanonische und nicht-kanonische BDZ-Bindungsstellen der $GABA_A$Ren. Verschiedene Hinweise deuten auf die Wichtigkeit der bevorzugten Bindung von BDZs an α2-enthaltende $GABA_A$Ren für die schmerzlindernden Effekte, anstelle von α1$GABA_A$Ren, welche für den sedativen Effekt verantwortlich sind. Ich habe deshalb ausgewählte BDZs charakterisiert, um Präparate mit einem grossen α2/α1 Selektivitätsverhältnis zu finden. Die kanonischen BDZ-Bindungsstellen werden durch Kopplung von einer von vier α-Untereinheiten (α1, α2, α3 oder α4) und der γ2-Untereinheit gebildet. Ich habe herausgefunden, dass N-Desmethylclobazam (NDMC), das wichtigste Abbauprodukt des lizenzierten Epilepsie-Medikaments Clobazam (CBZ), eine höhere Selektivität für den anti-hyperalgetischen, α2-enthaltenden $GABA_A$-Rezeptor als das klassische BDZ Diazepam (DZP) hat. Unter den sechs getesteten Stoffen wies Imidazotriazin TPA023B das beste α2/α1 Verhältnis auf. Diese Beobachtungen stimmten mit früheren Ergebnissen überein. Die nicht-kanonischen BDZ Bindungsstellen sind aus ‘nicht-γ2’-haltigen $GABA_A$Ren aufgebaut. Von den drei γ-Untereinheiten erreicht nur die γ1-Untereinheit in der Wirbelsäule ein potentiell relevantes Expressionsniveau, während im Gehirn γ2 höher exprimiert ist. Zusätzlich gibt es Hinweise dafür, dass noch andere Faktoren zusätzlich zu den α-Untereinheiten zum pharmakodynamischen Profil von BDZs beitragen. Ich habe deshalb die Aktivität von mehreren nicht oder wenig sedativen BDZs auf γ1-enthaltende $GABA_A$Ren untersucht. Das Benzodiazepin HZ-166 zeige hierbei eine höhere Wirksamkeit bei γ1$GABA_A$Ren als bei γ2$GABA_A$Ren. Verglichen mit anderen BDZs zeigte HZ-166 die höchste Wirksamkeit an γ1$GABA_A$Ren bei Konzentrationen unter dem Sättigungspunkt. HZ-166 könnte deshalb genutzt werden um die anti-hyperalgetischen Effekte von spinalen γ1$GABA_A$Ren zu untersuchen.

Zusammengenommen beschreibt diese Dissertation einen neuen Mechanismus für die spezifische pharmakologische Interaktion mit «schmerzrelevanten» GlyRen, sowie ein neues Werkzeug zur Analyse von γ1$GABA_A$Ren in der spinalen Schmerzkontrolle

Abbreviations and chemical names

2,6-DTBP , 2,6-ditertbutyl phenol

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF, brain-derived neurotrophic factor

BDZ, benzodiazepine-binding site ligand

CBZ, clobazam, 7-Chloro-1-methyl-5-phenyl-1,5-benzodiazepine-2,4(3H)-dione

CCL2, chemokine (C-C motif) ligand 2

CCI, chronic constriction injury

CFA, complete Freund's adjuvant

CGS-20625, 5,6,7,8,9,10-Hexahydro-2-(4-methoxyphenyl)cyclohepta[b]pyrazolo[3,4-d]pyridin-3(2H)-one

CGRP, calcitonin gene-related peptide

CVLM,caudal ventrolateral medulla

COX, cyclooxygenase

DMCM, methyl1-6,7-dimethoxy-4-ethyl-beta-carbonile-3-carboxylate

DRG, dorsal root ganglia

DZP, diazepam

EP2, prostaglandin E receptor type 2

$GABA_AR$, γ-aminobutyric acid type A receptor

GAD, glutamate decarboxylase

GlyR, glycine receptor

HDAC, histone deacetylase

HZ-166, ethyl 8-ethynyl-6-(pyridine-2-yl)-4H-benzo[f]imidazo]1,5-a][1,2,4]triazol-3-carboxylate

IB4, isolectin B4

IPSC, inhibitory postsynaptic current

KCC2, potassium/chloride cotransporter type 2

L-838,417. 3-(2,5-Difluorophenyl)-7-(1,1-dimethylethyl)-6-[(1-methyl-1H-1,2,4-triazol-5-yl)methoxy]-1,2,4-triazolo[4,3-b]pyridazine

LTD, long term depression

LTP, long term potentiation

NDMC, N-desmethyl clobazam

NGF, nerve growth factor

NK1, neurokinin 1

NMDA, N-methyl D-aspartate

NS11394, [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile]

PAG, periaqueductal grey

PGE_2, prostaglandin E2

PKA, protein kinase A

RVM, rotsroventromedial medulla

SP, substance P

TP003, 5-fluoro-2-[4-fluoro-3-[8-fluoro-7-(2-hydroxypropan-2-yl)imidazo[1,2-a]pyridin-3-yl]phenyl]benzonitrile

TPA023B, 6,2′-difluoro-5′-[3-(1-hydroxy-1-methylethyl)imidazo[1,2-b][1,2,4]triazin-7-yl]biphenyl-2-carbonitrile

TRP, transient receptor potential

1. General Introduction

A BRIEF HISTORY OF PAIN

When Aristotle numbered the five senses, he left out Pain. He, and his master Plato, thought that pain is not a sensory experience, but an emotional one, placing the heart as the central organ for this experience. Pain seemed to be carried by external beings or spirits entering through the injury commanded by gods in a sort of punishment. This supernatural conception of pain was then refuted during the Renaissance by René Descartes. He imagined the human body as a machine. He proposed that pain is a mechanical activation of a single cable from the site of the disturbance directly to the brain. Therefore, the understanding of pain moved from a mystical experience to a natural, internal one; from the heart to the brain. The first half of the 20th century witnessed the developing of two new theories based on the work from Max von Frey and Alfred Goldscheider. On one hand, the 'sensory theory' holding that different nerves convey different types of information, being pain a specific modality like hearing and vision; and on the other hand, the 'pattern theory', stating that any kind of sensory stimulus being intense enough would elicit pain. The concept of the specificity theory is still alive and builds the basis of the labelled line hypothesis (Craig, 2003), which proposes that dedicated sensory neurons and neuronal projections exist for the different somatosensory sensations. A more complex concept proposing an interaction of nociceptive and non-nociceptive signals has been developed by Patrick Wall and Ronald Melzack in their famous gate control theory of pain. This theory states that neurons located in the *substantia gelatinosa* of the spinal dorsal horn function as a gate control system that modulates the outcome of a painful stimulus to the brain. This concept had a major impact on our current understanding of pain.

GENERAL FEATURES OF PAIN

The international society of the study of pain (IASP) defines pain as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Bonica, 1979). In children born with congenital disability to experience pain, severe injuries often go unnoticed and can lead to repeated and potentially irreversible tissue damage and even death (Cox et al., 2006). Given the fact that pain also has an affective and emotional component, the perception of pain is subjective and also influenced by many factors. For instance, injured athletes are often not aware of pain until the match is over. Therefore, pain is not only a sensory experience, but rather the result of a complex processing of diverse neural signals by the brain including emotional cues.

Nature has provided us with the ability to perceive acute noxious threats, triggering protective reflexes and increasing our awareness. Usually, intense mechanical (noxious pressure, noxious movements, etc.) or thermal stimuli (noxious heat, noxious cold) are necessary to activate the nociceptive system, and when stimulated, *physiologic nociceptive pain* is elicited. However, during inflammation processes or tissue injury, *pathophysiologic nociceptive pain* is evoked. This inflammatory pain is characterized by mechanical and/or thermal allodynia and hyperalgesia. Allodynia refers to pain evoked by innocuous stimuli as light touch, whereas hyperalgesia describes a state of increased sensitivity to stimuli that are sensed as painful under normal conditions. In the course of inflammatory pain, the nociceptive system undergoes significant changes, but its functions are intact. On the other hand, pain can result from damage of neurons of the somatosensory system. This type of pain is called *neuropathic* pain.

THE NOCICEPTIVE SYSTEM

To better understand the neural processes underlying the perception of pain, it is necessary to have a look at the nociceptive neuroanatomy. Many organs in the periphery, including skin, muscles and joints, possess specialized sensory receptors, called primary nociceptors, which are activated by noxious stimuli. They convert noxious stimuli into electrical signals delivering these signals into the spinal cord or brain stem. The information is further conveyed to higher CNS areas where the conscious experience of pain takes place (Figure 1).

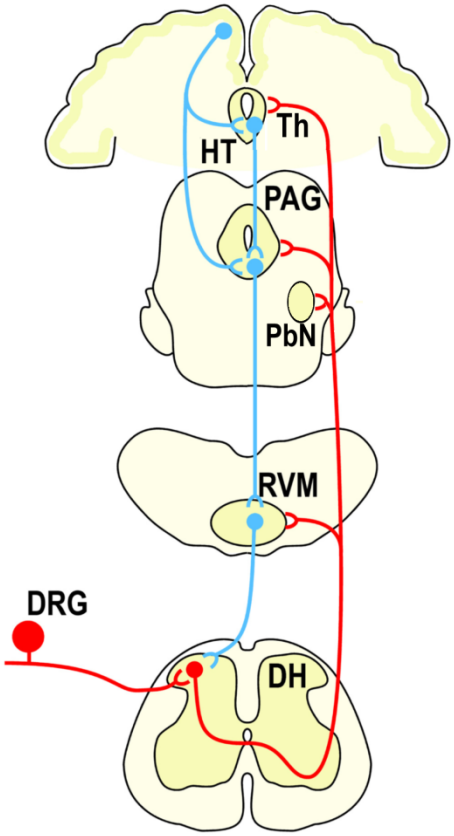


Figure 1. **Schematic diagram of the spinal pain pathway**. Nociceptive signals are conveyed to the central nervous system by the nociceptors (high-threshold primary sensory neurons), whose somata are located in the dorsal root ganglia (DRGs). They make contact to dorsal horn (DH) neurons in the spinal cord or brain stem, which in turn transmit the nociceptive information to supraspinal centers, including the rostral ventromedial medulla (RVM), the parabrachial nucleus (PbN), the periaqueductal grey (PAG) and thalamus (Th). Neurons in the cortex make contact with the hypothalamus (HT) and PAG, which control the RVM. From the RVM, descending tracts eventually control spinal nociception. Modified from Zeilhofer et al., 2015

Peripheral nociceptors

Exposure to a noxious stimulus depolarizes the primary nociceptors terminals in the periphery through the opening of specific ion channels (Waxman and Zamponi, 2014). This depolarization, if strong enough, opens voltage-gated sodium channels (e.g. $Na_v1.8$), which then elicit action potentials travelling along the fiber from their terminal in the periphery, through the DRG to the dorsal horn of the spinal cord. Here, the central terminals of the primary nociceptors make synapses with second order neurons located in different layers of the spinal dorsal horn.

Innocuous information is carried by myelinated Aβ fibers. Most of the nociceptors are thinly myelinated Aδ fibers or unmyelinated C fibers. These fibers differ in their electrical properties. C fibers have a conduction velocity of 0.5 – 2 m/s, which is slower than the myelinated Aδ fibers (12 – 30 m/s). Aβ fibers exhibit conduction velocities between 30 - 70 m/s

(Markopoulos, 2010). These distinct velocities reveal the type of pain that is driven by these fibers. For instance, Aδ fibers account for the sharp and localized pain sensation, while C fibers elicit a slower, dull and more diffuse sensation.

By the use of various neurochemical markers, C fibers are further subdivided into peptidergic, which express calcitonin gene-related peptide (CGRP) or substance P (SP), and non-peptidergic, which are characterized by the binding of isolectin B4 (IB4). C fibers may have distinct physiological roles. Peptidergic C fibers have been implicated in thermal nociception (McCoy et al., 2013), whereas non-peptidergic C fibers in mechanical (Bogen et al., 2008) and also thermal (Malin et al., 2006) nociception. The peripheral endings of these nociceptors harbor specific receptors and ion channels, which mediate different sensory information. For instance, sensitivity to heat partially depends on the transient receptor potential (TRP) vanilloid type 1 and 2 (V1/2), cold sensing arises mainly through activation of TRP menthol 8 (TRPM8) and/or TRP Ankyrin 1 (TRPA1) (Julius and Basbaum, 2001). In the dorsal spinal cord, these different primary afferent fibbers terminate in a characteristic pattern ordered in layers.

Spinal cord

The Swedish neuroscientist Bror Rexed identified in the early 1950s a system of ten distinct layers in the grey matter of the spinal cord (Rexed, 1952) (Figure 2A). The particular organization of the dorsal horn of the spinal cord goes along with defined primary afferents input patters (Figure 2B). Lamina I is the most superficial layer of the spinal cord. This lamina mainly harbors projection neurons, which can be identified by the expression of neurokinin 1 receptor (NK1-R). Neurons within lamina I are responsive to noxious stimuli, via Aδ and peptidergic C fibers. Virtually all of the neurons in lamina II are interneurons, e.g. their axons remain in the spinal cord and arborize locally. Interneurons can be divided into two main classes: excitatory (glutamatergic) and inhibitory (GABAergic and glycinergic). The inhibitory population in lamina II is one third of the total number present in this lamina. Neurons in lamina II are also responsive to either noxious or innocuous stimuli. Neurons located in the most dorsal part (lamina IIo) receive inputs from peptidergic C fibers. In contrast, non-peptidergic C fibers make contact with neurons located in the mid-region of lamina II (Braz et al., 2005). The most ventral part (lamina IIi) is characterized by interneurons expressing protein kinase C isoform γ (PKCγ), which are targeted by myelinated non-nociceptive primary afferents (Neumann et al., 2008). Laminae III and IV contain a mixture of local interneurons and supraspinal projection neurons. Many of these neurons are primary responsive to innocuous stimuli (via Aβ fibers). Neurons in lamina V respond to a wide variety of noxious stimuli via direct Aδ and Aβ (monosynaptic) inputs and indirect C fiber (polysynaptic) inputs.

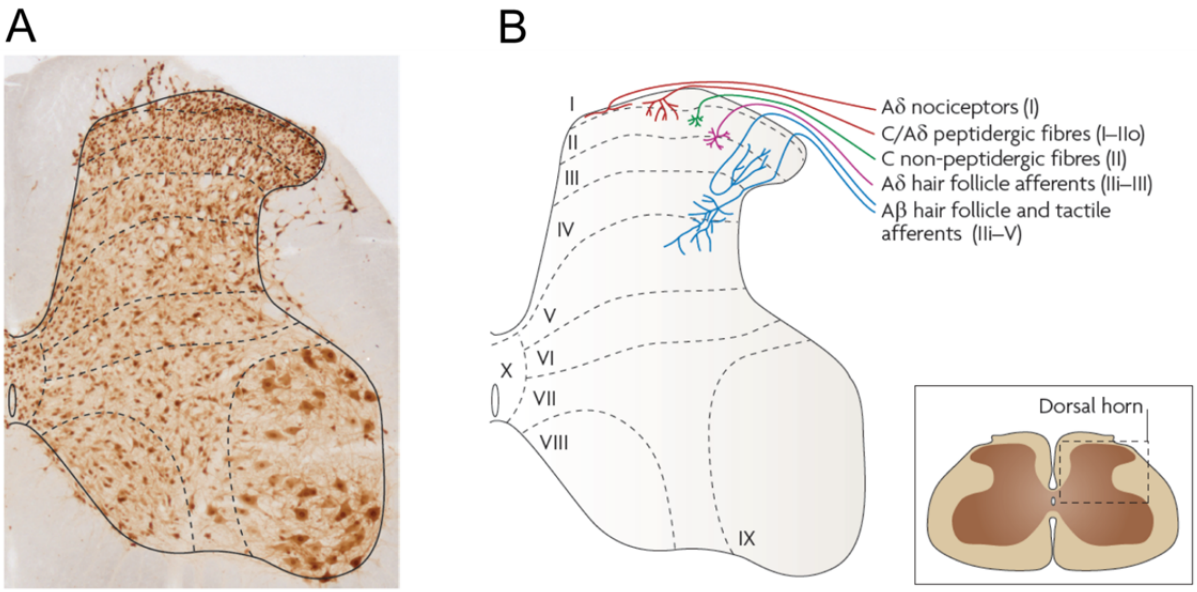


Figure 2. Architectonic organization of the spinal cord. **(A)** Rat spinal cord neurons labeled with NeuN. Dashed lines correspond to the Rexed division of laminar boundaries. **(B)** Scheme of the laminar termination patter of primary afferents. Reproduced from (Todd, 2010), with permission.

Ascending Pathways, supraspinal pain territories

Projection neurons are mainly present in lamina I and scattered throughout lamina III-VI. Many of these neurons project their axons crossing the midline and travel rostrally in the contralateral white matter to terminate in various brainstem and thalamic nuclei, establishing the spinoreticulothalamic and spinothalamic tracts, respectively.

Using anterograde and retrograde tracing it has been revealed that main targets for lamina I projection neurons include the caudal ventrolateral medulla (CVLM), the periaqueductal grey matter (PAG), the nucleus of the solitary tract (NTS), and certain nuclei in the thalamus (Gauriau and Bernard, 2004)

From these brainstem and thalamic loci, nociceptive information travels towards cortical territories. Unlike any other sensory modalities, a single specific brain area cannot be attributed to pain sensation (Apkarian, 2013). Nevertheless, some regions have been described to be involved in certain features of pain. For instance, the somatosensory cortex may be involved in the sensory-discriminative properties (Auvray et al., 2010); the anterior cingulate and insular cortex are associated to emotional aspects (Gao et al., 2004); and both the insula and prefrontal cortex are involved in cognitive processing of pain (Kong et al., 2006). However, some other regions not related to pain processing, like basal ganglia and cerebellum, have been shown to be also activated (Basbaum et al., 2009).

Descending pathways

From supraspinal territories, descending fibers can project onto the dorsal spinal cord modulating spinal pain processing (Gebhart, 2004). This regulation is primarily done by

monoaminergic pathways from several supraspinal sites (Millan, 2002). Classical studies have suggested an important role of PAG on descending pathways-mediated analgesia (Reynolds, 1969, Yaksh and Rudy, 1978). Following research demonstrated that the PAG is part of a central circuit that controls nociceptive transmission via a relay in the rostral ventromedial medulla (RVM) (Fields and Heinricher, 1985). This connection is critical for descending pain modulation (Fields et al., 1991). RVM connects to the spinal cord through serotoninergic and GABAergic neurons. Their axons project to dorsal horn lamina I, II and V; as stated previously, these laminae are targets of nociceptive primary afferents. According to morphological and electrophysiological evidence, RVM neurons regulate nociception via several mechanisms including direct inhibition of projection neurons, reduction of neurotransmitter release from nociceptive fibers, increase of excitation of inhibitory interneuron, and inhibition of excitatory interneurons (Todd, 2010). However, functional studies have demonstrated that brainstem modulatory mechanisms are bidirectional, in that not only inhibit nociception but also elicit pain facilitation (reviewed in Porreca et al., 2002). For instance, RVM has been shown to be implicated in hyperalgesia and chronic pain as part of a possible positive feedback loop stimulated by noxious input.

CELLULAR AND MOLECULAR MECHANISMS OF PATHOLOGICAL PAIN

Pain therapy is relevant to most fields of medicine since pain is a co-morbidity of numerous diseases. It is estimated that 20 % of the European population suffer from chronic pain, where osteoarthritis pain and low back pain are the leading causes (Breivik et al., 2006). Two thirds of chronic pain patients report that their therapy against pain is not successful and, in many cases, it leads to depression and inability to work. The Institute of Medicine in the USA reports that the number of patients suffering of chronic pain outnumbers patients suffering from cancer, diabetes and heart disease combined (Scholz and Woolf, 2002). This brings along a monetary burden of about $ 635 billion in medical treatment and loss of productivity. Therefore, intense pain research is necessary to improve patients' well-being. A major goal of pain research is to elucidate the neuronal mechanisms underlying the generation and maintenance of pain. As described previously, pain is determined by neurophysiological mechanisms in the nociceptive system as well as by psychological and socio-cultural influences. Thus, advanced understanding of the processes integrating the clinical symptoms of pain is a key step towards identifying the mechanisms that can be targeted for pain treatment. Chronic pathological pain can be initiated or maintained at peripheral and/or central regions.

Peripheral sensitization

During the course of inflammation or after tissue damage, primary nociceptors undergo plastic changes. They not only develop lower sensory thresholds to different mechanical and/or thermal stimuli, but also start to fire action potentials at higher frequencies (Schaible and Richter, 2004). During an inflammatory process, resident cells, such as keratinocytes and mast cells, also release inflammatory mediators, including prostaglandins, protons, ATP, bradykinin, nerve growth factor (NGF), interleukins, histamine and serotonin, which directly activate and modulate numerous ion channels and receptors located in nociceptive nerve fibers. These pro-inflammatory molecules trigger a *hyperalgesic priming* state (reviewed in Reichling and Levine, 2009). A very well-characterize inflammatory mediator is NGF. NGF activates TrkA receptors (receptor tyrosine kinase A, tropomyosin-related kinase A) leading to the activation of several signaling cascades, which in turn, will increase the expression of certain genes involved in nociceptors hypersensitivity, for instance, TRPV1, bradykinin receptors, sodium channels, substance-P and CGRP (Woolf, 1996, Fjell et al., 1999). Peripheral sensitization, therefore, leads to primary hyperalgesia, which is a localized sensitization at the area of tissue damage. The increase in nociceptive transmission from the periphery induces secondary changes in the central nervous system (CNS).

Central sensitization

Central sensitization results from changes in excitability of CNS neurons being fundamental for the generation of allodynia, secondary hyperalgesia and spontaneous pain (Latremoliere and Woolf, 2009, Sandkuhler, 2009, Kuner, 2010). Secondary hyperalgesia refers to a form of sensitization occurring in an area surrounding the site of inflammation and nociceptor activation. This secondary hyperalgesia is not accompanied by increased in nociceptor excitability but rather caused by changes in central pain processing.

Central sensitization was demonstrated in human volunteers by intradermal capsaicin injection. It was then established that heat pain at the zone of injection, was carried by C fibers, whereas the secondary mechanical hyperalgesia and allodynia was transferred to the CNS by Aβ mechanoreceptors and low threshold myelinated fibers, respectively (Torebjork et al., 1992). A large number of studies have identified that central sensitization contributes to several pain phenotypes, including rheumatoid arthritis (RA), osteoarthritis (OA), fibromyalgia (FM) and neuropathic pain (for review see Woolf, 2011).

This change in central pain processing can be caused by several cellular and molecular mechanisms, including development of long-term potentiation (LTP) of excitatory synapses and a reduction of GABAergic and or glycinergic inhibitory neurotransmission (disinhibition).

Increase of spinal synaptic strength

Synaptic strength refers to the magnitude of a post-synaptic response, in terms of currents or potentials, upon activation of pre-synaptic neurons. This synaptic strength can be modified by the quality of pre-synaptic inputs, either by high or low frequency activity of those inputs. In the hippocampus, it has very well described that a high frequency stimulation of pre-synaptic neurons (or fibers) can trigger LTP (Bliss and Lomo, 1973). However, these plastic changes in synaptic response have not only been seen in brain areas, but also in the spinal cord. Spinal LTP can be triggered by intense C fiber input (in a use use-dependent manner) to lamina I projection neurons (Sandkuhler et al., 1997, for review see Sandkuhler, 2009). Therefore, this mechanism of synaptic strength at spinal cord has been correlated to learning and memory formation in the hippocampus. As in the hippocampus, spinal LTP also depends on N-methyl D-aspartate receptors (NMDARs) and T-type voltage-gated calcium channels (Ikeda et al., 2003), and involves the insertion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) into glutamatergic synapses. This strengthened spinal synaptic transmission can also be induced *in vivo* by capsaicin or formalin injection in peripheral territories, as well as peripheral inflammation and nerve injury (Randic et al., 1993, Ikeda et al., 2003). It is quite likely that similar forms of plasticity exist in supraspinal territories, including synapses in the cerebral cortex (Li et al., 2010, Zhuo, 2011)

Disinhibition

About one-third of in the spinal dorsal horn are inhibitory. These neurons release GABA and/or glycine to promote inhibition of the post-synaptic cells by inducing a chloride conductance through ionotropic $GABA_A$ and glycine receptors, respectively, and therefore hyperpolarizing the cell. It is also worth noting that GABA can activate metabotropic $GABA_B$ receptors, which trigger inhibition through facilitating the opening of G protein-coupled inwardly-rectifying potassium (GIRK) channels in the post-synaptic cell, and by inhibiting voltage-gated calcium channels in the pre-synaptic neuron.

The concept of inhibition in spinal pain processing was firstly proposed in the gate control theory of pain (Melzack and Wall, 1965). This theory, in its original version, suggested that inputs arriving to the dorsal spinal cord from low-threshold mechanoreceptors and high-threshold nociceptors interact with intrinsic inhibitory neurons to open or close the pain gate. Therefore, they proposed a pivotal role of inhibitory interneurons in the spinal control of nociceptive signal propagation. About 20 years later, the first experimental proof implicating an endogenous inhibitory control by GABAergic and glycinergic neurotransmission was performed by testing the effects of spinal application of specific blockers of $GABA_A$Rs and glycine receptors. Animals spinally injected with theses antagonists responded with symptoms of hyperalgesia and of allodynia along with spontaneous pain (Beyer et al., 1985,

Roberts et al., 1986, Sivilotti and Woolf, 1994), as described previously, signs of central sensitization. A recent publication revisited the gate control theory of pain and demonstrated the crucial role of dorsal horn glycinergic interneurons in pain processing (Foster et al., 2015).

In pathological pain states GABAergic and glycinergic inhibition can get compromised in neuropathic pain as well as in inflammatory pain through a variety of mechanisms which I will describe below.

Activation of microglia by mediators, such as chemokine (C-C motif) ligand 2 (CCL2), released from the central terminals of primary afferents is one initiating event leading to diminished inhibitory neurotransmission in response to peripheral nerve damage (Zhang and De Koninck, 2006). Brain-derived neurotrophic factor (BDNF), released by activated microglia, downregulates the expression of the potassium/chloride cotransporter KCC2, which plays an important role on maintaining low intracellular chloride concentration. This increase of chloride concentration decreases (or even reverses) the hyperpolarizing action GABA and glycine due to the shift in the chloride reversal potential towards more depolarized voltages (Coull et al., 2003, Coull et al., 2005).

After nerve injury, an incomplete loss of the GABA synthesizing enzyme GAD65 in the dorsal spinal cord occurs (Moore et al., 2002, Lorenzo et al., 2014). The consequence of this is a depletion of GABA in inhibitory boutons, leading to diminished inhibition. Moreover, in persistent inflammatory and neuropathic pain states, the transcription of the gene encoding for GAD65 (*GAD2*) is epigenetically suppressed through histone deacetylase (HDAC)-mediated histone hypoacetylation (Zhang et al., 2011)

Secondary hyperalgesia and allodynia caused by intraplantar injection of capsaicin in mice is at least partially mediated by a reduction of glycinergic and GABAergic inhibition. Excitatory nociceptive-specific interneurons receive less inhibitory post-synaptic currents (IPSCs) caused by spinal production of endocannabinoids (eCB) and the subsequent activation of cannabinoid 1 (CB1) receptors located on the presynaptic inhibitory terminal. Upon intense glutamatergic C fiber input, endocannabinoids, retrogradely-released from presumed excitatory interneurons, activate the CB1 receptors located at the inhibitory pre-synaptic neuron, which in turn reduce the release of inhibitory neurotransmitter (Pernia-Andrade et al., 2009), ultimately promoting disinhibition.

Diminished synaptic inhibition in the spinal dorsal horn also occurs in the course of peripheral inflammation. It has been shown that after peripheral inflammation, glycinergic neurotransmission is reduced (Muller et al., 2003). Prostaglandins are important mediators of inflammation and contribute also to central sensitization. In the spinal cord, prostaglandin E_2

(PGE_2) is produced by the inducible cyclooxygenase 2 (COX-2) from arachidonic acid. Spinal pronociceptive actions of PGE_2 occur through PGE_2 receptors of the EP_2 subtype expressed on excitatory interneurons (Reinold et al., 2005). EP_2 receptors subsequently activate protein kinase A (PKA) through an increase of cyclic adenosine monophosphate (cAMP). PKA phosphorylates glycine receptors containing the GlyRs α3 subunit (α3GlyRs), rendering these receptors less responsive to synaptically released glycine (Ahmadi et al., 2002, Muller et al., 2003, Harvey et al., 2004) (Figure 3). This decrease in GlyR activation induces mechanical and thermal hyperalgesia. Moreover, mice lacking the GlyRα3 subunit ($GlyR\alpha3^{-/-}$) show reduced sensitization and faster recovery from peripheral inflammation by Zymosan A and complete Freund's adjuvant (CFA). This phenotype is specific for inflammatory pain and does not occur in neuropathic pain models, where mechanical and thermal hyperalgesia remain unchanged. PGE_2-dependent disinhibition therefore appears to play a role primarily in inflammatory hyperalgesia (Zeilhofer et al., 2012).

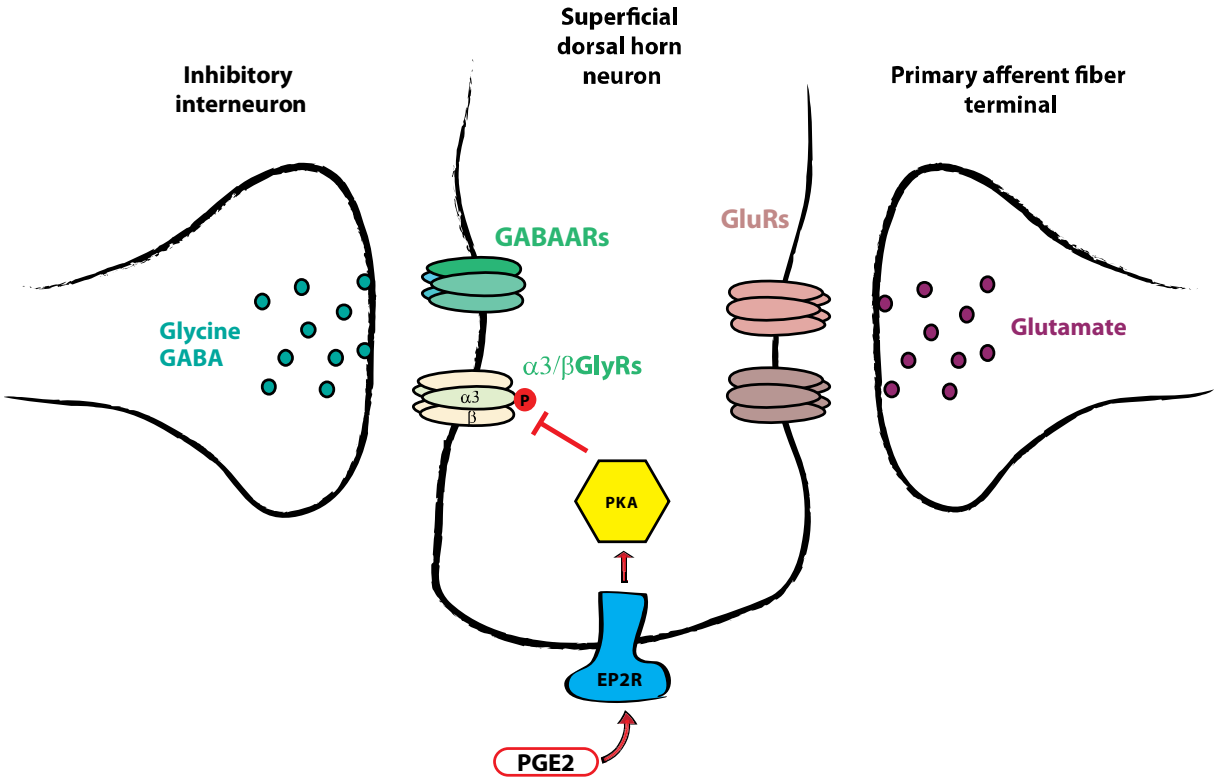


Figure 3. Schematic representation of the PGE_2-dependent spinal disinhibition in inflammatory pain. Peripheral inflammation induces the expression of COX-2 and synthesis of PGE_2 in the spinal cord. PGE_2 activates EP2 receptor and promotes the activity of PKA through an increase of cAMP. PKA then phosphorylates and inhibits GlyRs containing the α3 subunit (GlyRα3). Modified from Zeilhofer et al., 2012.

All the mechanisms mentioned above indicate that pathological pain syndromes (either from inflammatory origin or neuropathic cause) merge onto diminished GABAergic and/or glycinergic inhibition in the dorsal spinal cord. This disinhibition mimics the major symptoms of persistent pain conditions in human such as hyperalgesia, mechanical allodynia, and on-

going pain. Therefore, a pharmacological enhancement of GABAergic or glycinergic inhibition in the spinal dorsal horn should thus have a beneficial effect on pathological pain syndromes. It has been shown that spinal application of the classical benzodiazepine-site agonist (BDZ) diazepam (DZ), which facilitates the action of GABA at $GABA_A$Rs, reverses pathological pain sensitization in different rodent models of inflammatory and neuropathic pain (Knabl et al., 2008, Di Lio et al., 2011, Ralvenius et al., 2015). A thorough analysis of the molecular basis of GlyR and $GABA_A$R modulation in the spinal cord along with an evaluation of the value of GlyR and $GABA_A$R modulators as potential novel analgesics should therefore be of significant interest.

FAST INHIBITORY NEUROTRANSMITTER RECEPTORS

In the spinal cord, the two major inhibitory neurotransmitters are glycine and GABA. Ionotropic glycine and $GABA_A$ receptors belong to the Cys-loop superfamily of ligand-gated ion channels, which also includes nicotinic acetylcholine receptors and ionotropic serotonin (5-HT3) receptors. Both glycine and $GABA_A$ receptors are permeable to chloride ions and to a lesser extent to bicarbonate (Figure 4). Glycine and $GABA_A$ receptors are formed by five subunits, each of them harboring four transmembrane segments (Figure 4A)

Glycine receptors

Glycine receptors are, together with $GABA_A$ receptors, a major inhibitory neurotransmitter receptor in the spinal cord, brainstem and selected supraspinal areas including the retina (Araki et al., 1988). The repertoire of subunits is restricted to only four α subunits (α1 - α4) and one β subunit (Lynch, 2004) (Figure 4B). In humans, no functional GlyRα4 subunit expressed due to a premature stop codon and several amino acid exchanges ((Laube et al., 2002, Simon et al., 2004) and personal communication by Robert J. Harvey). GlyR α subunits can form functional homopentameric glycine-sensitive ion channel. This is not the case for β subunit, which, when expressed alone, does not produce glycine-gated currents. Heteropentameric glycine receptors composed of α and β subunits, form functional receptors. Their single channel conductance is less than that of homomeric receptor channels (Burzomato et al., 2004). The subunit stoichiometry has been subject of debate (for review, see Lynch, 2009), however, recent evidence suggests that the conformation of heteromeric α/β glycine receptors is 2α:3β (Grudzinska et al., 2005). The proper clustering of glycine receptors at post-synaptic sites occurs via a direct interaction between the β subunit and the cytoplasmic anchoring protein, gephyrin (Fritschy et al., 2008).

During development, most of glycine receptors in the CNS are homomeric α2-containing receptors, which are subsequently replaced by α1β or α3β heteromers (Takahashi et al., 1992). Homomeric α1 or α3 receptors, which cannot bind gephyrin, are more likely

expressed at extrasynaptic site. However, evidence from electrophysiological recordings on spinal neurons suggests that most extrasynaptic glycine receptors also exhibit a heteromeric α/β conformation (Beato and Sivilotti, 2007). Nevertheless, homomeric GlyRs might be found on pre-synaptic terminals controlling neurotransmitter release (Turecek and Trussell, 2001, Jeong et al., 2003, Deleuze et al., 2005).

In the CNS, the α1 subunit is most abundant among the GlyR α subunits (Becker et al., 1998), and the β subunit is also widely distributed throughout the spinal cord, retina and brain (Malosio et al., 1991). Therefore, most of glycine receptors at synapses are α1β heteropentamers. GlyR α3 subunits have been mapped to the retina and the spinal cord). Interestingly, in the spinal cord, expression of α3GlyRs is restricted to superficial dorsal horn (Harvey et al., 2004), where nociceptive fibers terminate. α3GlyRs might therefore be important elements of spinal inhibitory pain control.

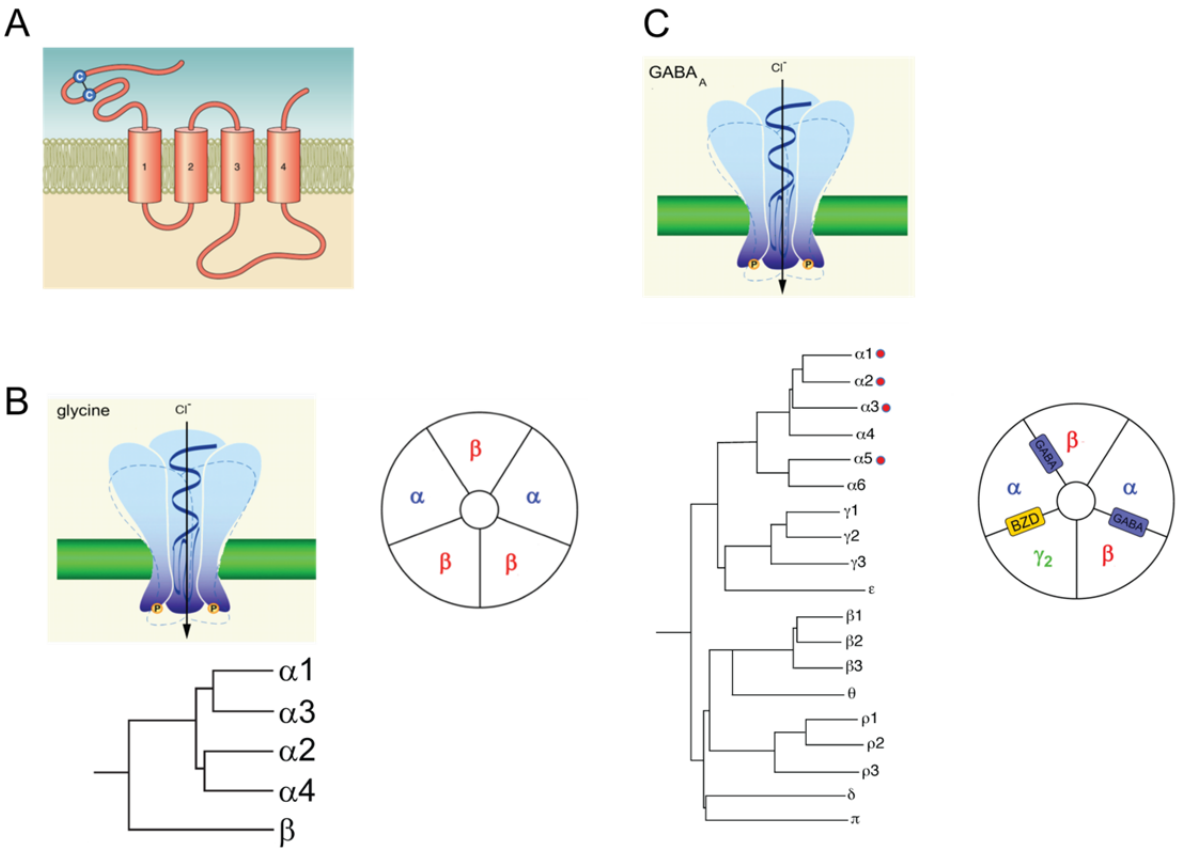


Figure 4. Inhibitory neurotransmitter receptors in the spinal cord. (**A)** Diagram depicting the subunit 4-transmembrane segments of glycine and $GABA_A$ receptors. B-C) Schematic representation of glycine receptor **(B)** and of $GABA_A$Rs **(C)** indicating the subunit repertoire and conformation in the receptor complex. Red dots indicate all benzodiazepine-sensitive α subunits.

$GABA_A$ receptors

Mammalian $GABA_A$Rs are heteropentameric ligand-gated ion channels assembled from a repertoire of 19 different subunits: α1 - α6, β1 - β3, γ1 - γ3, δ, ε, π, θ and ρ1 - ρ3 (Figure 4C).

Most of $GABA_A$Rs are formed with a stoichiometry of two α, two β, and one γ subunit. This configuration mediates phasic inhibition in the CNS. In the spinal cord, the most abundant configuration is α2/3, β3, γ2, whereas in the brain, most of $GABA_A$Rs are composed of α1β2γ2 (Wisden et al., 1991, Laurie et al., 1992, Bohlhalter et al., 1996). The subunit arrangement determines important features of the receptor, including the affinity to GABA, channel kinetics, pharmacological properties and location targeting (e.g. synaptic or extrasynaptic). For instance, the γ2 subunit is required for the synaptic clustering of most of $GABA_A$R subtypes (Essrich et al., 1998), whereas receptors incorporating the δ subunit (together with α4/6 and β) are localized extrasynaptically, where they mediate tonic inhibition (for review, see Belelli et al., 2009).

Upon release from presynaptic terminals, GABA binds to the interface between α and β subunit. $GABA_A$Rs assemble in such a configuration that allows to set two GABA binding site per receptor (Figure 4C). Beside the GABA-binding sites, $GABA_A$Rs also possess sites for modulation of endogenous neuromodulators, such neurosteroids, and exogenous compounds like BDZs , barbiturates, alcohols and anesthetics (Sieghart, 1995, Sigel and Buhr, 1997, Belelli et al., 1999, Rudolph and Mohler, 2006). Even though BDZs facilitate the action of GABA at $GABA_A$Rs and are widely used clinically for their sedative, anxiolytic and anticonvulsive effects, BDZs do not exert pain-relieving actions in patients or in human experimental pain models (for review, see van Tulder et al., 2003; but also see Besson et al., 2015). The BDZ-binding site is located in the interface between γ2 and α1, α2, α3 or α5 (Figure 4C) (Mohler et al., 2001). The presence of γ1 or γ3 also allows to the binding of BDZs, but with strongly reduced affinities (Benke et al., 1996).

STRATEGIES FOR RESTORING GLYCINERGIC AND GABAERGIC INHIBITION

Evidence for modulators acting on GlyRs

New evidence suggests that glycine receptors can be modulated by a number of compounds, including cannabinoids and general anaesthetics (for a review, see Yevenes and Zeilhofer, 2011a).

Endogenously produced cannabinoid receptor ligands (endocannabinoids), such as N-arachidonyl-ethanolaminde (anandamide, AEA) and 2-arachidonyl-glycerol (2-AG), can directly modulate GlyRs (Lozovaya et al., 2005, Hejazi et al., 2006, Yevenes and Zeilhofer, 2011b). Some structurally unrelated compounds such as phyto-cannabinoids, namely Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), also exert modulating activity at GlyRs. A recent study (Xiong et al., 2011) has provided the first evidence of the role of GlyR subunits on the antihyperalgesic effects of THC. However, one important point is that cannabinoid-related compounds lack specificity (Oz, 2006). To overcome this, Xiong and

colleagues (Xiong et al., 2011) developed THC-derivatives with reduced affinity to the parental's natural targets, the cannabinoid receptor (CB) 1, and CB2. Further work from the same group (Xiong et al., 2012a, Xiong et al., 2012b) established that cannabinoids acting on GlyRs suppress inflammatory and neuropathic pain, without major psychoactive side effects and analgesic tolerance. Furthermore, it was demonstrated that the cannabinoid-mediated antihyperalgesic effects in pathological pain are mediated by a α3GlyR-dependent mechanism (Xiong et al., 2012a).

Volatile and intravenous anaesthetics also modulate GlyRs. Many volatile anaesthetics can potentiate homomeric α1GlyRs, such as isoflurane, enflurane, halothane and sevoflurane (Downie et al., 1996, Mascia et al., 1996, Krasowski and Harrison, 1999, Yamakura et al., 2001). Although it has been shown that α2GlyRs are also modulated (Harrison et al., 1993), there is no evidence on the effects of volatile anaesthetics on α3GlyRs. Intravenous anaesthetics, such as propofol, also modulate glycinergic function (Mascia et al., 1996, Mihic et al., 1997, Pistis et al., 1997, Wakita et al., 2016). The biological effects of propofol, however, appeared to be only by enhancing GABAergic function, rather than potentiating glycinergic inhibition (Sonner et al., 2003, Grasshoff and Antkowiak, 2004). Interestingly, new evidence has shown that propofol structural analogs, such as 4-bromopropofol and 2,6-di-tert-butylphenol, retain modulatory effects at glycine receptor sparing activity at $GABA_A$Rs (Krasowski et al., 2001, Ahrens et al., 2004, Ahrens et al., 2009, Eckle et al., 2014).

The modulation of glycinergic inhibition may represent a novel therapeutic strategy to reverse disinhibition. According to the evidence presented above, the search for specific modulator of spinal synaptic α3βGlyRs is a rational approach against inflammatory pain. Indeed, as discussed earlier, PGE_2 released upon inflammation "primes" α3βGlyRs, decreasing synaptic glycinergic current in spinal superficial dorsal horn. Compounds modulating specifically "primed" α3βGlyRs may, therefore, restore the glycinergic inhibition in inflammatory pain states, avoiding unwanted effects such as sedation.

Evidence for modulation of $GABA_A$Rs by BDZ

In humans, benzodiazepines can exert analgesic effects when applied intraspinally through a modulation of BDZ-sensitive $GABA_A$Rs (Tucker, 1985). However, when systemically applied, benzodiazepines can cause pain relief only at doses that, at the same time, cause strong sedation (Ralvenius et al., 2015; for review, see also Zeilhofer et al., 2009). To attribute the different benzodiazepine effects to identified $GABA_A$R subtypes, point mutated mice have been created, which carry a single histidine to arginine amino acid exchange in the α1, α2, α3 or α5-$GABA_A$R subunit. These mutations render the receptor insensitive to benzodiazepines (Rudolph and Mohler, 2006, Knabl et al., 2008). Using this tool, it has

become clear that different $GABA_AR$ subunits contribute to distinct effects of BDZ. Sparing the α1-containing $GABA_ARs$, which are expressed preferentially in the brain cortex, the classical BDZ diazepam (DZP) produces profound analgesia without any sedation (Knabl et al., 2008, Ralvenius et al., 2015). Moreover, α2 and α3-$GABA_ARs$ have been shown to be the most relevant subtypes for BDZ-mediated antihyperalgesia after systemic DZP administration (Figure 5A) (Ralvenius et al., 2015). This characteristic feature matches well with the expression pattern of different α subunits in the superficial spinal dorsal horn (Figure 5B-E) (Bohlhalter et al., 1996, Paul et al., 2012). Therefore, sparing BDZ activity at α1, the antihyperalgesic effects of BDZs can be studied in absence of confounding sedative effects.

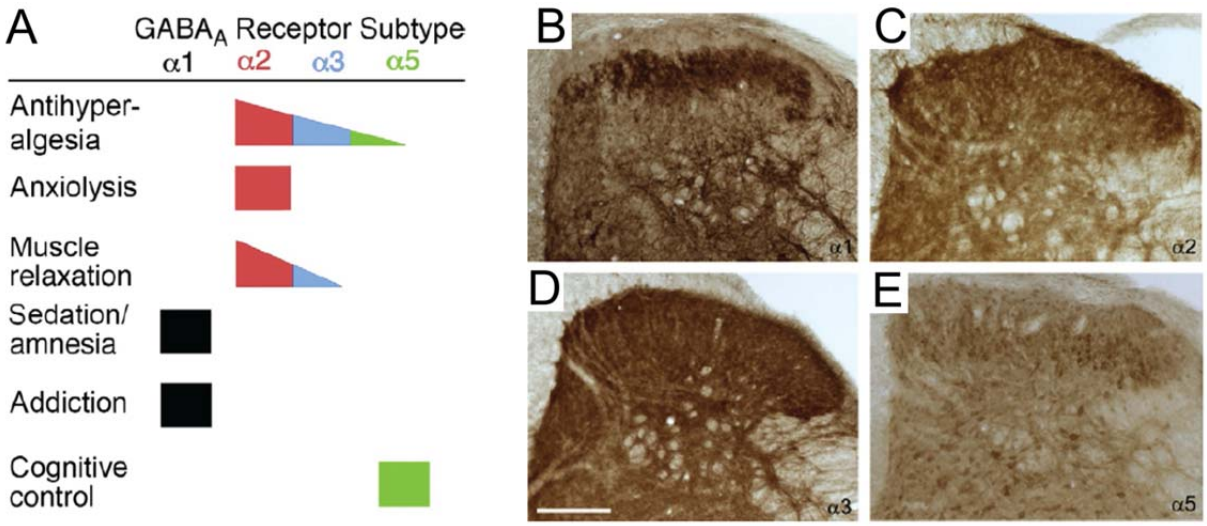


Figure 5. Contribution to antihyperalgesia and distribution of the BDZ-sensitive α subunits of the $GABA_ARs$ in the dorsal spinal cord. (A) Contribution of the different α subunits to spinal antihyperalgesia in comparison with other behavioral BDZ-mediated effects. **(B-E)** Expression pattern of α1 (B), α2 (C), α3 (D) and α5 (E). Scale bar 100 µm. Modified from Zeilhofer et al., 2015.

Several subtype-selective compounds with reduced activity at α1$GABA_ARs$ have been identified recently (Rudolph and Knoflach, 2011). For instance, NS11394, a compound that shows low intrinsic activity at α1$GABA_ARs$, exhibited antinociceptive activity at non-sedating doses in various inflammatory and neuropathic pain models (Mirza et al., 2008, Hofmann et al., 2012). L-838,417, which shows selectivity at α2, α3 and α5 and, was also efficacious against inflammatory and neuropathic pain. This compound lacks activity at α1-$GABA_ARs$, and it does not produce sedation (Knabl et al., 2008, Nickolls et al., 2011). HZ-166, a newly synthesized BDZ (Rivas et al., 2009) with better activity at α2/α1 than diazepam, exerts antihyperalgesic effects in different pain models with sedation only at high doses and no tolerance development (Di Lio et al., 2011, Paul et al., 2014).

This evidence suggests that α2$GABA_AR$-selective (α1-sparing) compounds might exert robust antihyperalgesia with low unwanted effects. Therefore, studying new compounds with

such a profile may provide new therapeutic approaches to restore inhibition in pathological pain states.

Evidence for modulation of $GABA_ARs$ by non-canonical BDZ-binding site ligands

Although classical BDZs require the γ2 subunit for full potentiation, γ1 and γ3 containing $GABA_ARs$ still exhibit some potentiation by BDZ-site ligands (Puia et al., 1991, McKernan et al., 1995, Khom et al., 2006, Baburin et al., 2008, Esmaeili et al., 2009). The substitution of phenylalanine at the position 77 to isoleucine in the γ2 subunit (γ2F77I) confers insensitivity to certain BDZs (Ogris et al., 2004). The generation of a mouse line carrying the γ2F77I mutation has led to a better understanding of benzodiazepine allosteric modulation on $GABA_ARs$ (Cope et al., 2004, Ogris et al., 2004, Cope et al., 2005, Leppa et al., 2005, Wulff et al., 2007, Ramerstorfer et al., 2010, Leppa et al., 2011a, Leppa et al., 2011b). Indeed, there is evidence of a functional expression of BDZ-sensitive γ1$GABA_ARs$ in γ2F77I histaminergic neurons, suggesting an *in vivo* role of non γ2-containing receptors on the modulation by BDZs (May et al., 2013). Additionally, evidence from our laboratory suggests that the BDZ-mediated spinal effect does not depend exclusively on the effect at γ2-containing $GABA_ARs$. Indeed, the γ2$GABA_AR$ BDZ-antagonist flumazenil reduced analgesic effect of DZP only at high concentrations when spinally applied (Knabl et al., 2008), indicating that a non-classical benzodiazepine site was targeted. Moreover, a non-BDZ compound DMCM, which antagonizes γ2$GABA_ARs$ but exerts agonistic effects at γ1$GABA_ARs$, produces antihyperalgesic actions in neuropathic and inflammatory pain models (Munro et al., 2008). Furthermore, while γ2 subunit is largely expressed throughout the brain, γ1 subunit is expressed in more restricted areas (Hortnagl et al., 2013). Therefore, compounds interacting selectively with γ1$GABA_ARs$ might add another level of selectivity when one wants to target spinal GABAergic pain control.

2. Aims

The overall aim of this thesis was to explore opportunities for pharmacological restoration of compromised synaptic inhibition in the spinal dorsal horn in the context of chronic pain conditions. Two specific aims were defined which addressed GlyRs and $GABA_A$Rs, respectively:

Aim 1: Characterize the capacity of the non-anesthetic propofol derivative 2,6-DTBP as a GlyR modulator *in vitro* and assess the contribution of GlyR modulation to its analgesic effect *in vivo*.

2,6-DTBP has previously been reported to be devoid of activity at $GABA_A$Rs, to potentiate GlyR and to possess analgesic efficacy *in vivo*.

Aim 2: Characterize the modulatory effects of typical and atypical BDZs at canonical and non-canonical binding sites of the $GABA_A$Rs.

In this part of my project I pursued two research directions. (1) I compared the *in vitro* pharmacological profile of typical benzodiazepines (such as diazepam) with those of atypical benzodiazepines that either differed from classical compounds by their structure or showed reduced sedative properties than their classical counter parts. (2) Some previous findings of our group suggest that part of the analgesic effects of spinally applied benzodiazepines might occur through non-canonical benzodiazepine binding sites that might for example contain γ subunits different from γ2. There is some evidence that the γ1 subunit might be expressed in the spinal dorsal horn at higher levels than in the brain. I therefore tested the pharmacological profile of several benzodiazepine site ligands with analgesic efficacy on heterologously expressed $GABA_A$Rs to compare their actions on γ2 and γ1 $GABA_A$Rs.

3. Experimental Section

Chapter I: Phosphorylation State-Dependent Modulation of Spinal Glycine Receptors Alleviates Inflammatory Pain[+]

[1,2]*Mario A. Acuña, [1,3]*Gonzalo E. Yévenes, [1,2]William T. Ralvenius, [1,2,4]Dietmar Benke, [1,2]Alessandra Di Lio, [3]Cesar O. Lara, [3]Braulio Muñoz, [3]Carlos F. Burgos, [5]Gustavo Moraga-Cid, [5]Pierre-Jean Corringer, [1,2,4,6]Hanns Ulrich Zeilhofer

[1]Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

[2]Neuroscience Center Zurich (ZNZ), Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

[3]Department of Physiology, Faculty of Biological Sciences, University of Concepcion, Barrio Universitario S/N, 4070386, Concepcion, Chile

[4]Drug Discovery Network Zurich (DDNZ), Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

[5]Channel-Receptors Unit, Institute Pasteur, 25 rue du Dr. Roux, F-75015, Paris, France

[6]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH) Zurich, Vladimir-Prelog-Weg 4, CH-8093 Zurich, Switzerland

* M.A.A. and G.E.Y. contributed equally to this work

Correspondence to:

Dr. Hanns Ulrich Zeilhofer, Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. Phone: +41 44 63 55 912, Email: zeilhofer@pharma.uzh.ch, or

Dr. Gonzalo E. Yévenes, Department of Physiology, Faculty of Biological Sciences, University of Concepcion, Barrio Universitario S/N, 4070386, Concepcion, Chile. Phone: +56 41 266 13 90. Email: gyevenes@udec.cl

[+] This work has been published in *The Journal of Clinical Investigation*. (Acuña MA et al., 2016)

Contribution of Mario A. Acuña: I performed all electrophysiological recordings of this study except the single channel recordings, and the *in vivo* experiments.

ABSTRACT

Diminished inhibitory neurotransmission in the superficial dorsal horn contributes to chronic pain. In inflammatory pain, this reduction occurs at least partially through a prostaglandin E_2 (PGE_2) and protein kinase A (PKA)-dependent phosphorylation of a specific subtype of glycine receptors (GlyRs) containing α3 subunits. Here, we show that the non-anesthetic propofol derivative 2,6-di-tert-butylphenol (2,6-DTBP) reverses this inflammation-mediated dis-inhibition through a specific interaction with heteromeric $\alpha\beta$GlyRs containing phosphorylated α3 subunits. Electrophysiological analyses of mutated receptors show that 2,6-DTBP interacts with a conserved phenylalanine residue in the membrane-associated stretch between transmembrane regions 3 and 4 of the GlyR α3 subunit. In native spinal cord tissue, 2,6-DTBP modulated synaptic GlyR currents only after priming with PGE_2, consistent with molecular modeling data that suggest that in heteromeric $\alpha3\beta$GlyRs this site is accessible to 2,6-DTBP only after PKA-dependent phosphorylation. *In vivo*, 2,6-DTBP reduced inflammatory hyperalgesia in a α3GlyR-dependent manner. Our data thus establish that potentiation of GlyR function is a promising strategy against chronic pain and that 2,6-DTBP has a unique pharmacological profile favoring an interaction with GlyRs primed by peripheral inflammation.

INTRODUCTION

Chronic pain states are associated with complex molecular and cellular changes in the peripheral and central nervous system (Basbaum et al., 2009; Zeilhofer et al., 2012; Luo et al., 2014). An increasing body of evidence indicates that diminished inhibitory GABAergic and glycinergic neurotransmission at the level of the spinal dorsal horn makes an important contribution to chronic pain states (Zeilhofer et al., 2012; Sandkühler 2009; Coull et al., 2003). A variety of mechanisms have been identified which differentially contribute to diminished synaptic inhibition in various inflammatory and neuropathic pain states (Basbaum et al., 2009; Zeilhofer et al., 2012). In case of inflammatory pain, a reduction in glycinergic inhibition occurring through a PGE_2- and PKA-dependent phosphorylation of a specific subtype of glycine receptors (i.e. those containing the α3 subunit, α3GlyR) has a major role (Harvey et al., 2004). Restoring activity of synaptic α3GlyR through positive allosteric modulators may therefore constitute a mechanism-based therapeutic approach against chronic inflammatory pain.

Despite the importance of GlyRs in a variety of physiological processes, GlyR pharmacology is still poorly developed and only few GlyR modulators are available at present (Lynch 2009; Yévenes & Zelhofer, 2011a; Laube et al., 2002). Some of these compounds such as several propofol derivatives modulate GlyR at nanomolar or even subnanomolar concentrations (de la Roche et al., 2012; Eckle et al., 2012), but none of them are fully specific for GlyRs and many of them also modulate the function of other ion channels (Yévenes & Zelhofer, 2011a). This lack of specificity has hampered establishing causal links between molecular and therapeutic actions (Xiong et al., 2011; Xiong et al., 2012; Yang et al., 2008; Zhang et al., 2013).

Using molecular, electrophysiological and behavioral approaches, we explored the basis of GlyR allosteric modulation by the non-anesthetic propofol derivative 2,6-di-*tert*-butylphenol (2,6-DTBP). 2,6-DTBP potentiates currents through recombinant α1GlyRs (Ahrens et al., 2004) but has negligible activity at $GABA_A$ receptors ($GABA_ARs$) (Ahrens et al., 2009; Krasowski et al., 2001). Here, we show that 2,6-DTBP is a positive α3GlyR allosteric modulator, which effectively restores the loss of α3GlyR-mediated inhibition in inflammatory pain states at least partially through a selective potentiation of phosphorylated GlyRs containing α3 and β subunits. A specific phenylalanine residue in the α3 subunit appears critical for this potentiation. Thus, our results establish a novel mechanism for pharmacological intervention against inflammatory pain through phosphorylation-specific potentiation of spinal α3GlyR.

METHODS

Reagents. 2,6-DTBP and PTX were obtained from Sigma-Aldrich. PGE_2, bicuculline, strychnine, and H89-dihydrochloride were purchased from Tocris (Bristol, UK). D-APV and CNQX were obtained from ANAWA (Wangen, Switzerland). 2,6-DTBP and PGE_2 were dissolved in DMSO at 100 µM and kept at -20°C.

Electrophysiology on recombinant GlyRs. Recombinant GlyRs were transiently expressed in HEK293T cells (originally obtained from Atcc, LGC Standards GmbH, Wesel, Germany) using lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). One µg of DNA / 35 mm dishes was used for transfection of α1 and α3 subunits together with 0.5 µg of an eGFP expression plasmid to allow identification of the transfected cells. In experiments on $\alpha\beta$ heteromeric GlyRs, plasmids encoding α and β subunits were transfected at a ratio of 1:15. To confirm the expression of heteromeric GlyRs, picrotoxin (PTX)-sensitivity was routinely monitored (Lynch, 2009) (Figure S1). All recordings were made 18 - 36 hrs after transfection. The cDNA encoding the GlyRs has been described previously (Yévenes & Zeilhofer, 2011b) Mutations were inserted using the QuickChange site-directed mutagenesis kit (Agilent Technologies) or by an external supplier (Mutagenex, NJ, USA). Proper sequences of all constructs were confirmed by full-length sequencing.

Glycine-evoked whole-cell currents were recorded at room temperature (20 - 24°C) at a holding potential of -60 mV using the patch-clamp technique. Patch electrodes (3 - 4 MΩ) were pulled from borosilicate glass and filled with (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.4), 4 $MgCl_2$, 0.5 GTP and 2 ATP. The external solution contained (in mM) 150 NaCl, 10 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4), and 10 glucose. Recordings were performed with a HEKA EPC-7 amplifier and Patch Master v2.11 software (HEKA Elektronik, Lambrecht-Pfalz, Germany). Glycine was applied via a manually controlled gravity fed application system with an ID of 200 µm ID positioned 50 - 120 µm from the recorded cell. Glycine EC_{10} values for each GlyR studied were obtained experimentally after successive application of 1, 10, 30, 60, 100, 200, 500 and 1000 µM glycine (see also Table S2). The concentration-response curve parameters (EC_{50} and Hill coefficients, n_h) were obtained from the curve fits of normalized concentration-response to the equation $I_{gly} = I_{max}[gly]^{nH}/([gly]^{nH} + [EC50]^{nH})$. The mean maximal current (I_{max}) was determined by applying 1 mM glycine. 2,6-DTBP was first dissolved in DMSO (100%) at a concentration of 100 mM and subsequently diluted in several steps to the final concentration on the day of the experiment. Before use solutions were vigorously shaken for 60 min. Maximum DMSO concentration was 0.3%. 2,6-DTBP was co-applied with glycine, without pre-application. Single channel recordings from transfected cells were done as described (19, 28). All recordings were performed in the cell-attached configuration at 19 - 21°C with thick-walled borosilicate glass pipettes (final

resistance of 10 - 15 MΩ). The extracellular solution contained (mM): 20 Na-gluconate, 102.7 NaCl, 2 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 Hepes, 20 TEA-Cl, 15 sucrose, and 14 glucose, pH 7.4. The pipette solution was filled with the same extracellular solution but containing glycine (100 - 150 µM) or glycine plus 2,6-DTBP (10 µM). Single channel currents were recorded with an RK-400 patch-clamp amplifier (Bio-Logic) at a pipette potential of +60 mV. Recordings were pre-filtered at 10 kHz and were digitized to a computer using a Digidata 1400 (Molecular Devices) and pClamp 10 (Axon Instruments).

Molecular modeling and docking. Because most of the structural data available on GlyRs do not include the TM3-TM4 intracellular domain, we modeled the α3 subunit as previously described for α1GlyR (Burgos et al., 2015) using the ab initio technique with QUARK (Xu & Zhang, 2012). The full α3GlyR subunit was created by homology modeling using GluCl [PDB: 2RIF] (Hibbs & Gouaux, 2011) as template in Modeller 9v13 (Eswar et al., 2006). Additional refinement was performed using the α1GlyR-GLIC structure (Moraga-Cid et al., 2015). The final model was obtained after energy minimization with a conjugate gradient protocol in the software MacroModel (version 9.9, Schrödinger, LLC, New York, NY, 2012). The resulting helical conformation of the C-terminal region of the TM3-TM4 intracellular domain (MA-stretch) of α3GlyR was similar to the recently resolved structure for the 5-HT_3 receptor (Hassaine et al., 2014) and to the proposed TM3-TM4 intracellular domain of the α1GlyR subunit (Burgos et al., 2015). Docking protein-ligand was performed using the model of α3 subunit and the 2,6-DTBP structure available in the ZINC database [ID: ZINC01681254] (Irwin et al., 2012). An initial complex was created with Glide (version 5.9, Schrödinger, LLC, New York, NY, 2013) using a receptor grid centered on residue F388 of α3GlyR. Analysis of the interface by the same software including structural and energetic parameters generated a docking score (Friesner et al., 2006). Additionally, a second energy calculation MM-GBSA was performed using Prime (version 3.2, Schrödinger, LLC, New York, NY, 2013) to predict a theoretical ΔG_{bind}. Taken together, the docking score and ΔG_{bind} improved the description of the interaction between α3GlyR and 2,6-DTBP. The β subunit was modeled using the α1GlyR model (Burgos et al., 2015) and interfaces α3-α3 and α3-β were constructed based on the α1GlyR pentamer [PDB: 2M6B] (Mowrey et al., 2013). The α3GlyR F388 mutant was created using BioLuminate (version 1.1, Schrödinger, LLC, New York, NY, 2013) and consequent dockings were performed under the same conditions described above. All images were created using PyMol (version 1.5, DeLano Scientific LLC).

In situ proximity ligation assay (in situ PLA). PGE_2-induced phosphorylation of the α3 GlyR subunit was analyzed using the in situ PLA (Leuchowius et al., 2010). cDNAs of GlyR α3 and EP2, or GlyR α3(S246A) and EP2 were transfected into HEK293T cells grown on poly-lysine-coated coverslips using the PEI (polyethylenimine) method. Two days after

transfection, cells were incubated in 1 μM PGE_2 for 30 min. After fixation in 4% PFA for 30 min cells were permeabilized in 0.2% Triton/PBS. Phosphorylation of α3GlyR was tested with the in situ PLA using antibodies directed against α3GlyR (1:50, Millipore cat. # AB15014) and phosphoserine (1:5, Millipore cat. #05-1000X). The cells were incubated with the primary antibodies overnight and processed for in situ PLA using the Duolink kit (Sigma-Aldrich) as described previously (Zemoura & Benke, 2014). Images (as z-stack with a 0.8 μm z-interval) were taken with a Zeiss 510 Meta confocal laser scanning microscope using a Zeiss 100x plan-fluar objective (N.A. = 1.4). Quantification of in situ PLA signals was done by counting individual PLA fluorescence spots using ImageJ (NIH). The optical sections of each image stack were summed into one image, median filtered (r = 1.0 pixels) and subjected to automated spot detection. The number of spots was then normalized to the cell area.

RT PCR. Six lumbar spinal cords were rapidly removed from euthanized adult wild-type and GlyRα3$^{-/-}$ mice. mRNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen no.205311). Expression levels of GlyR α1 and β subunits relative to β-actin were determined using commercially available detection assays (Integrated DNA Technologies, Leuven, Belgium, catalog number Mm.PT.58.9052470 for *glra1*, Mm.PT.58.29236025 for *glrb*, and Mm.PT.58.33257376.gs for *actb*).

Recordings in spinal cord slices. Transverse spinal cord slices were prepared from 2 to 3-week-old vGAT::ChR2-eYFP mice (Zhao et al., 2011) and GlyRα3 deficient (GlyRα3$^{-/-}$) mice (Harvey et al., 2004) of either sex. Slices (400 μm thick) were kept in oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) containing (in mM): 120 NaCl, 5 HEPES, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 glucose (pH 7.35) at 35°C for 1 hr. Slices were then transferred to the recording chamber continuously perfused with oxygenated aCSF at a flow rate of 1.5 - 2.0 ml/min. Superficial dorsal horn neurons were visually identified using infrared gradient contrast equipment. Recordings in neurons obtained from vGAT::ChR2 mice were made from excitatory neurons identified by the absence of a light-induced photocurrent. Recordings in neurons from GlyRα3$^{-/-}$ mice were made from randomly chosen lamina II neurons. Whole-cell patch-clamp recordings were made at room temperature at a holding potential of -60 mV using a HEKA EPC-10 amplifier and Patch Master v2.11 software (HEKA Elektronik, Lambrecht-Pfalz, Germany). Patch pipettes (3.5 - 4.5 MΩ) were filled with internal solution containing: (in mM) 120 CsCl, 10 EGTA, 4 $MgCl_2$, 0.5 GTP, 2 ATP, and 10 HEPES (pH 7.30 adjusted with CsOH). QX-314 (5 mM) was added to block voltage-activated Na^+ currents in the recorded cell. Once the whole-cell mode was established, the cell was allowed to stabilize for 2 - 5 min. Light-evoked inhibitory post-synaptic currents (IPSCs) were elicited by whole-field blue light (473 nm) illumination (4 ms duration) at a frequency of 0.1 Hz. Glycinergic or GABAergic light-evoked

IPSCs were isolated using bicuculline (10 μM) or strychnine (1 μM), respectively. In GlyRα3$^{-/-}$ mice, glycinergic IPSCs were evoked by electrical field stimulation at a frequency of 0.1 Hz (100 μs, 3 - 30 V) using a glass electrode (5 – 7 MΩ) filled with standard extracellular solution and placed about 50 μm from the recorded neuron (6). Gly-IPSCs were isolated using CNQX (5 μM), D-APV (50 μM) and bicuculline (10 μM). After 3 - 5 min of baseline recording, 2,6-DTBP (100 μM) or PGE_2 (1 μM) were added to the bath solution for 8 - 10 min. To examine the role of PKA-dependent phosphorylation, slices were continuously superfused with H89 (5 μM). At the end of each recording, strychnine (1 μM) or bicuculline (10 μM) were added to confirm the glycinergic or GABAergic nature of the recorded IPSCs.

Animals. Breeding pairs of GlyRα3$^{-/-}$ mice (Harvey et al., 2004)(6) were kindly provided by Dr. Robert J. Harvey, Dr. Ulrike Müller and Dr. Heinrich Betz (all the Max-Planck-Institute for Brain Research, Max-von-Laue Strasse 4, D-60438 Frankfurt, Germany). vGAT::ChR2-eYFP mice (29) were provided by Dr. Guoping Feng, McGovern Institute for Brain Research at MIT, 43 Vassar Street, Cambridge, MA 02139. GlyRα3$^{-/-}$ mice and vGAT::ChR2-eYFP BAC transgenic mice were maintained on a C57BL/6 background. All mice were group housed with standard 12-hr light/dark cycle and food and water available *ad libitum*.

Behavioral tests Antihyperalgesic properties of 2,6-DTBP (90 mg/kg i.p.) were studied in 7 - 12 week old mice subjected to the zymosan A and Complete Freund's Adjuvant (CFA) models of inflammatory hyperalgesia (Harvey et al., 2004; Meller & Gebhart, 1997). Mice were injected subcutaneously with either zymosan A (0.06 mg in 20 μl saline) or CFA (1:2 diluted in saline) into the plantar side of the left hind paw. To study the effects of 2,6-DTBP on neuropathic pain, mice were subjected to the chronic constriction injury (CCI, (Bennet & Xie, 1988)). In brief, three loose (5-0 silk) ligatures were put around the left sciatic nerve proximal to the trifurcation. Mice that showed signs of paralysis or did not develop significant hypersensitivity were excluded from subsequent experiments. Mechanical and thermal nociceptive sensitivities were determined using electronic von Frey filaments and the plantar test, respectively. 2,6-DTBP was dispersed in saline/0.05% TWEEN20 (vehicle), sonicated on ice and injected intraperitoneally (i.p.) (see also (Tibbs et al., 2013)). Sensitivities of the ipsilateral (inflamed or nerve-injured) paw and the contralateral control paw were measured alternately and at least four measurements were taken per mouse for all time points. Antihyperalgesia was quantified for the time interval of 30 - 60 (for zymosan A model and CCI) or 60 – 90 min (for CFA) post-drug injection when the drug effect was maximal, and expressed as percent maximum possible analgesic effect (MPE). MPE = $(R_{post\text{-}drug} - R_{pre\text{-}drug})/(R_{baseline} - R_{pre\text{-}drug}) \times 100$, where R is the average response latency or threshold under baseline condition ($R_{baseline}$), after induction of inflammation but before drug injection ($R_{pre\text{-}drug}$), and after drug injection ($R_{post\text{-}drug}$). Accelerating rotarod performance (from 4 to 40 r.p.m.)

of wild-type mice was measured after 60 min of the administration of 2,6-DTBP or vehicle. Each mouse was tested three times. Mice were trained with 2 different training sessions on 2 consecutive days before testing. The locomotor activity assays were performed during the light phase of the day-night cycle. The animals were placed in individual circular enclosures (diameter 20 cm) equipped with 4 photocells. The locomotor activity was expressed as the total number of photocell interruptions recorded in a period of 6 hrs immediately after drug administration. The horizontal wire test (Bonetti et al., 1982) was performed to assess potential muscle relaxation. Mice were placed forepaws-first onto a 15 cm metal wire and scored according to whether or not they were able to grasp the wire with their hindpaws or not. Acute pain was measured in the so called pin-prick test using an injection needle applied to the plantar surface of the hindpaw without breaking the skin. The number of paw withdrawals out of three trials was calculated as a percent for each time point. The experimenter was blind to mouse genotype and drug treatment. Special care was taken to ensure equal numbers of age-matched male and female mice in all behavioral experiments.

Statistics. All data are displayed as mean ± standard error of means (SEM). Statistical comparisons were made with 2-tailed paired or unpaired t-tests or with ANOVA or repeated measures ANOVA followed by appropriate post hoc tests. p values < 0.05 were considered statistical significant.

Study approval. Permission for the animal experiments was obtained from the Tierversuchskommission of the canton of Zurich, Zurich, Switzerland (license numbers 135/2009 and 126/2012).

RESULTS

Conformation-specific potentiation of recombinant α3GlyR by 2,6-DTBP

We first assessed the effects of 2,6-DTBP on glycine-evoked currents through recombinant GlyRs expressed in HEK293T cells. Homopentameric GlyRs composed of α3 subunits (α3GlyR) were strongly potentiated by micromolar concentrations of 2,6-DTBP (Figure 1A-B). The efficacy of potentiation by 2,6-DTBP (determined at a 2,6-DTBP concentration of 100 μM and at the EC_{10} of glycine) was higher for α3GlyRs than for α1GlyRs (211 ± 30% versus 115 ± 19%, $p < 0.05$, $n = 6$, unpaired t-test). We next assessed the modulation of heteromeric αβGlyRs (as these receptors constitute most GlyRs at postsynaptic sites). The efficient expression of heteromeric GlyRs containing both α and β subunits was confirmed by their relative insensitivity to picrotoxin (PTX), which blocks homomeric GlyRs with higher potency than αβ heteromeric receptors (Figure S1). Compared to homomeric GlyRs, potentiation of heteromeric α3β or α1β GlyRs was significantly weaker (20 ± 10% and 30 ± 6% for α3β and α1βGlyRs, respectively, Figure 1A-C). We then evaluated the biophysical mechanisms underlying the allosteric modulation by 2,6-DTBP of α3GlyRs. Whole-cell recordings showed that 2,6-DTBP caused a leftward shift in the glycine concentration-response curve of α3GlyR. EC_{50} values were 206 ± 1.0 μM and EC_{50} = 76 ± 2.3 μM, under control conditions and in the presence of 100 μM 2,6-DTBP, respectively, $p < 0.001$, unpaired t-test (Figure 1D), suggesting an increase in the apparent affinity of the receptor for its agonist. 2,6-DTBP did not affect the maximal current amplitude elicited by 1 mM glycine (5.0 ± 1.0 nA versus 4.8 ± 1.1 nA, in the presence and absence of 100 μM 2,6-DTBP, respectively). Single channel recordings in the cell-attached configuration (Marabelli et al., 2013) showed that 2,6-DTBP significantly increases the ion channel open probability (control nPo = 0.20 ± 0.02 versus 2,6-DTBP nPo = 0.58 ± 0.10, paired t-test, $p < 0.01$, Figure 1E,F, Table S1) without apparent changes in the main conductance levels (control γ = 91.0 ± 1.20 pS versus 2,6-DTBP γ = 90.1 ± 0.92 pS, paired t-test, Figure 1E,F, Table S1).

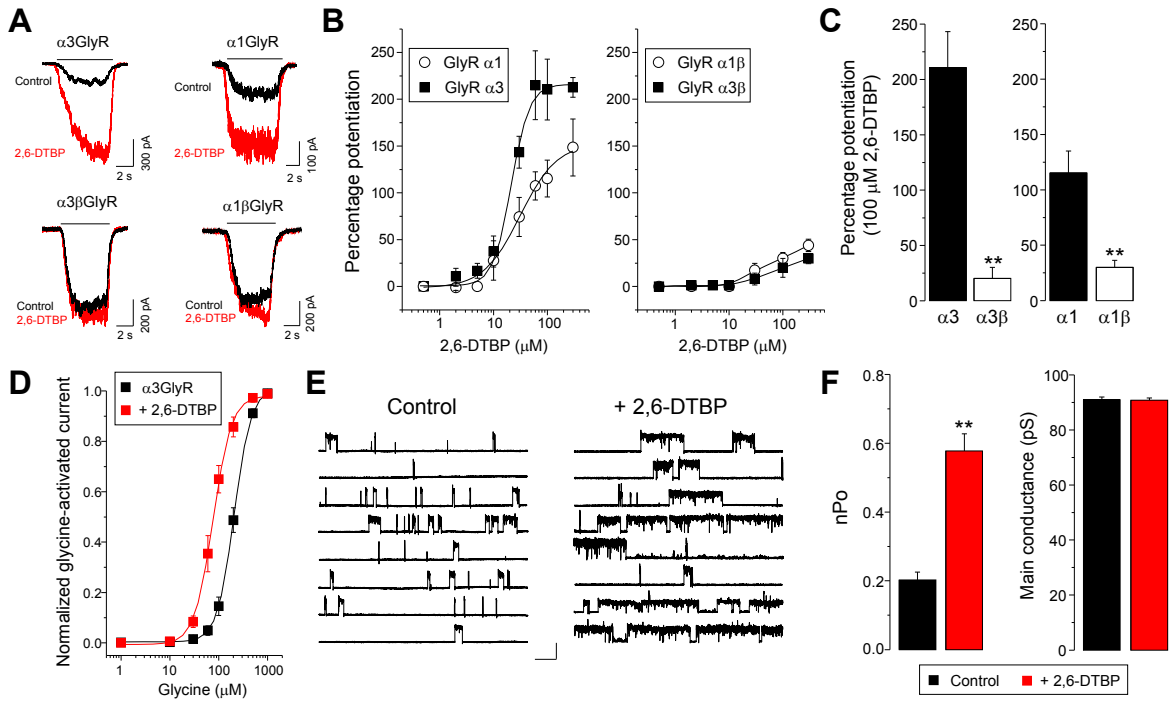


Figure 1. Modulation of recombinant GlyRs by 2,6-DTBP in HEK293T cells. (A) Example whole-cell current traces evoked by glycine (EC_{10}) in the absence or presence of 2,6-DTBP (100 μM) in homomeric α3 or α1GlyRs, and in heteromeric α3β or α1βGlyRs. **(B)** Concentration-response curves of 2,6-DTBP obtained with an EC_{10} of glycine in homomeric α3 or α1GlyRs, and in heteromeric α3β or α1βGlyRs. **(C)** Heteromeric α3/β and α1/β GlyRs are significantly less susceptible to modulation by 2,6-DTBP than homomeric α1 and α3GlyRs. **, $p < 0.01$, unpaired t-test. **(D)** Concentration-response curves of glycine in homomeric α3GlyR in the absence or in the presence of 2,6-DTBP (100 μM, red). **(E)** Single channel current traces recorded from membranes expressing α3GlyRs in the presence and the absence of 2,6-DTBP (10 μM). **(F)** 2,6-DTBP increases ion channel open probability (nPo) but not single channel main conductance. **, $p < 0.01$, paired t-test. Data are mean ± SEM from 6 - 10 cells (B-D) or 6 patches per group (F).

In order to explore the molecular determinants of α3GlyR modulation by 2,6-DTBP, we first focused on molecular sites in α1GlyR and α3GlyR involved in the modulation by propofol, the parent compound of 2,6-DTBP. Previous analyses of structure activity relationships have identified S267 in TM2, A288 in TM3 and F380 in the interface between the intracellular region and the TM4 domain as critical for the modulation of α1GlyR by propofol (Ahrens et al., 2008; Moraga-Cid et al., 2011; Lynagh & Laube, 2014). All three residues are conserved between α1GlyR and α3GlyR. Other studies have however found that mutation of two of these residues (S267 and A288) also severely interferes with GlyR function itself Mihic et al., 1997; Findlay et al., 2003). By contrast, mutation of F380 diminished propofol sensitivity without altering ion channel gating and conductance (Moraga-Cid et al., 2011). We therefore examined whether the mutation of phenylalanine to alanine in position 388 (F388A) would affect the sensitivity of α3GlyR to 2,6-DTBP. Whole-cell recordings revealed that the F388A

mutation did not change the sensitivity of homomeric α3GlyR to glycine (Figure 2A), but significantly impaired sensitivity to 2,6-DTBP. At 100 μM, 2,6-DTBP potentiated wild-type α3GlyR by 164 ± 20% (n = 12), whereas potentiation of F388A α3GlyR only reached 10 ± 9.0% (n = 10) (Figure 2B,C). To gain additional molecular insight, we analyzed the effects of 2,6-DTBP in single channel recordings of α3GlyR carrying the F388A mutation. 2,6-DTBP (10 μM) did not significantly modify activity of F388A mutant ion channels (control nPo= 0.18±0.05 versus 2,6-DTBP nPo = 0.19 ± 0.04, paired t-test), confirming the low sensitivity of the mutated receptor to modulation by 2,6-DTBP (wild-type = 192.8 ± 32.6% of nPo increase above control versus F388A = 5.7 ± 15.6%, unpaired t-test, $p < 0.001$, Figure 2D-F, Table S1). Additional analyses revealed that wild-type and F388A mutant GlyRα3 exhibited similar conductance levels (wild-type γ = 91.0 ± 1.20 pS versus F388A γ = 88.2 ± 1.82 pS, $p = 0.44$, unpaired t-test). These results confirm that the F388A mutation diminished the sensitivity of α3GlyRs to 2,6-DTBP without altering the ion channel function.

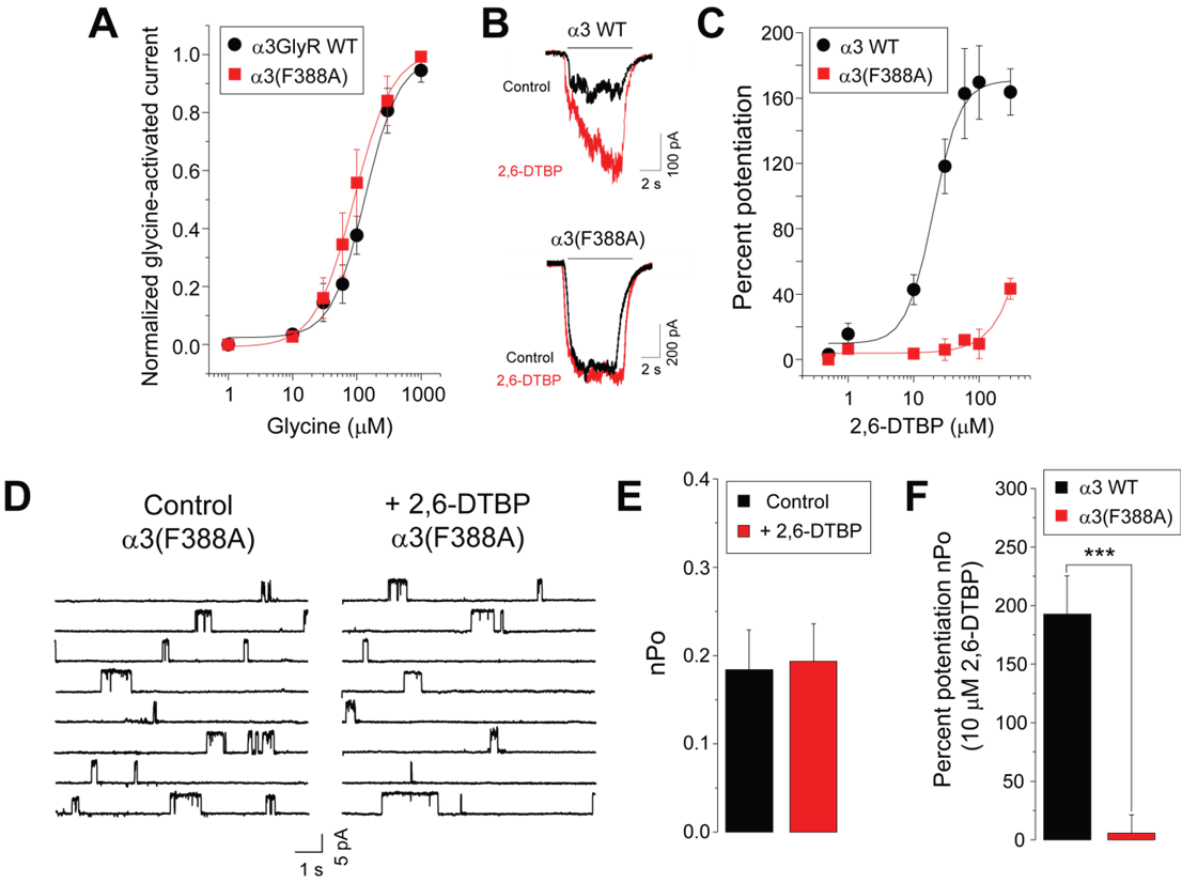


Figure 2. A molecular site for the actions of 2,6-DTBP on α3GlyRs. (A) Concentration-response curves of glycine in wild-type and (F388A) point mutated α3GlyRs. **(B)** Traces of glycine-activated whole-cell currents recorded from cells expressing wild-type or (F388A) point mutated α3GlyRs in the absence or presence of 2,6-DTBP (100 μM). **(C)** Concentration-response curves of 2,6-DTBP for wild-type and (F388A) point mutated α3GlyRs. **(D)** Examples of single channel recordings from membranes expressing point mutated (F388A) α3GlyRs in the presence or absence of 2,6-DTBP. **(E)** Ion channel nPo of the (F388A) α3GlyR in the absence or presence of 2,6-DTBP (10 μM). **(F)** Percent

change of nPo following the application of 2,6-DTBP in wild-type and (F388A) point mutated α3GlyRs. ***, $p < 0.001$, unpaired t-test. All data are mean ± SEM from 6 - 9 cells per group.

A molecular site for the actions of 2,6-DTBP on α3GlyR

The F388 residue required for modulation by 2,6-DTBP lies within the so called membrane associated (MA) stretch of the channel immediately upstream of the transmembrane region 4 (Carland et al., 2009). This site is close to a consensus site for PKA-dependent phosphorylation of GlyRα3 (serine 346) that has been previously implicated in inflammation-induced inhibition of synaptic GlyR currents in the superficial spinal dorsal horn (Harvey et al., 2004) (Figure 3A, note that the GlyRα1 subunit lacks this consensus site). In order to provide direct evidence for a PKA-dependent phosphorylation S346 in GlyRα3, we made use of the in situ proximity ligation assay (in situ PLA, (Leuchowius et al., 2010)). Incubation of HEK293T cells transiently transfected with wild-type GlyRα3 subunits and EP2 receptors led to a significant increase in the phosphorylation-dependent PLA signal after exposure to PGE_2 (1 µM). This increase was absent in point mutated GlyRα3(S346A) (Figure 3B,C). To explore a potential interaction of phosphorylation at S346 with α3GlyR modulation by 2,6-DTBP, we investigated the impact of S346 phosphorylation on the α3GlyR function and modulation and tested two point mutated receptors that mimic the phosphorylated (S346E) and non-phosphorylated (S346A) state. Electrophysiological recordings revealed that the phosphorylation state did not affect the sensitivity of homomeric or heteromeric α3GlyRs to their natural agonist glycine (Figure S2, Table S2). Likewise, homomeric GlyRs composed GlyRα3(S346A) or GlyRα3(S346E) did not differ much in their sensitivities to 2,6-DTBP (142 ± 23% in S346A versus 190 ± 42% in S346E, 100 µM 2,6-DTBP, Figure 3D-F). However, heteromeric GlyRs containing the phosphomimicking S346E mutation were significantly more sensitive to 2,6-DTBP than those containing the S346A mutation (S346A: 10 ± 8% versus S346E: 112 ± 31%, 100 µM 2,6-DTBP, $p < 0.01$, unpaired t-test). These results demonstrate that the heteromeric phosphorylated α3βGlyRs are more sensitive to modulation by 2,6-DTBP, indicating a conformation-selective modulation of α3βGlyRs. We then examined whether the mutation of F388 could affect the 2,6-DTBP sensitivity of phosphorylated α3GlyR. To this end, we studied double point mutated α3GlyRs containing both the S346E and the F388A mutation. The F388A mutation significantly attenuated the potentiation of homomeric α3(S346E)GlyRs and heteromeric α3(S346E)βGlyRs (Figure 3G). These data indicate that the F388 residue is critical for the potentiation of both homomeric α3 and heteromeric (phosphorylated) α3βGlyRs.

Given the key role of F388 in the actions of 2,6-DTBP on α3GlyR, we next asked whether this residue could be a critical part of an acceptor site for 2,6-DTBP. To this end, we developed a model of α3GlyR based on the crystal structures of GluCl and GLIC-GlyR (Hibbs & Gouaux, 2011). The results of this modeling were not only consistent with F388 being located in the MA-stretch close to transmembrane segment 4 (Figure 3H) but also provided insights into the molecular composition of the putative acceptor site for 2,6-DTBP. Three residues (F388, M384 and P381) appeared as particular relevant for hydrophobic interactions of 2,6-DTBP with α3GlyRs. The charged residues D382 and R385 complement the binding area. Molecular docking analyses revealed a favorable theoretical energy of interaction with this putative acceptor site (ΔG_{bind} = -42.09 kcal/mol, docking score = -2.133). The energy of interaction and the docking scores of 2,6-DTBP and of other propofol analogs with this molecular site correlate well with the functional modulation of wild-type α3GlyRs (Table S3). Interestingly, our modeling data suggest that the introduction of the F388A mutation causes a significant decrease in these parameters (ΔG_{bind} = -29.51 kcal/mol, docking score = -0.507), suggesting a direct relationship between the degree of potentiation of α3GlyR by 2,6-DTBP and the energy of interaction. Additional molecular modeling and simulations suggest that the β GlyR subunit also displayed a lower energy of interaction with 2,6-DTBP (ΔG_{bind} = -27.99 kcal/mol, docking score, 0.194), possibly because of the presence of an isoleucine at position 388 (i.e. I443 in the β subunit) and a poor structural homology with the α subunits in these particular regions (Figure S3). Overall, these results identified F388 as a pivotal residue for the actions of 2,6-DTBP on α3GlyR and suggest the presence of a 2,6-DTBP acceptor site in the MA-stretch of α3 that is not present within the β subunit.

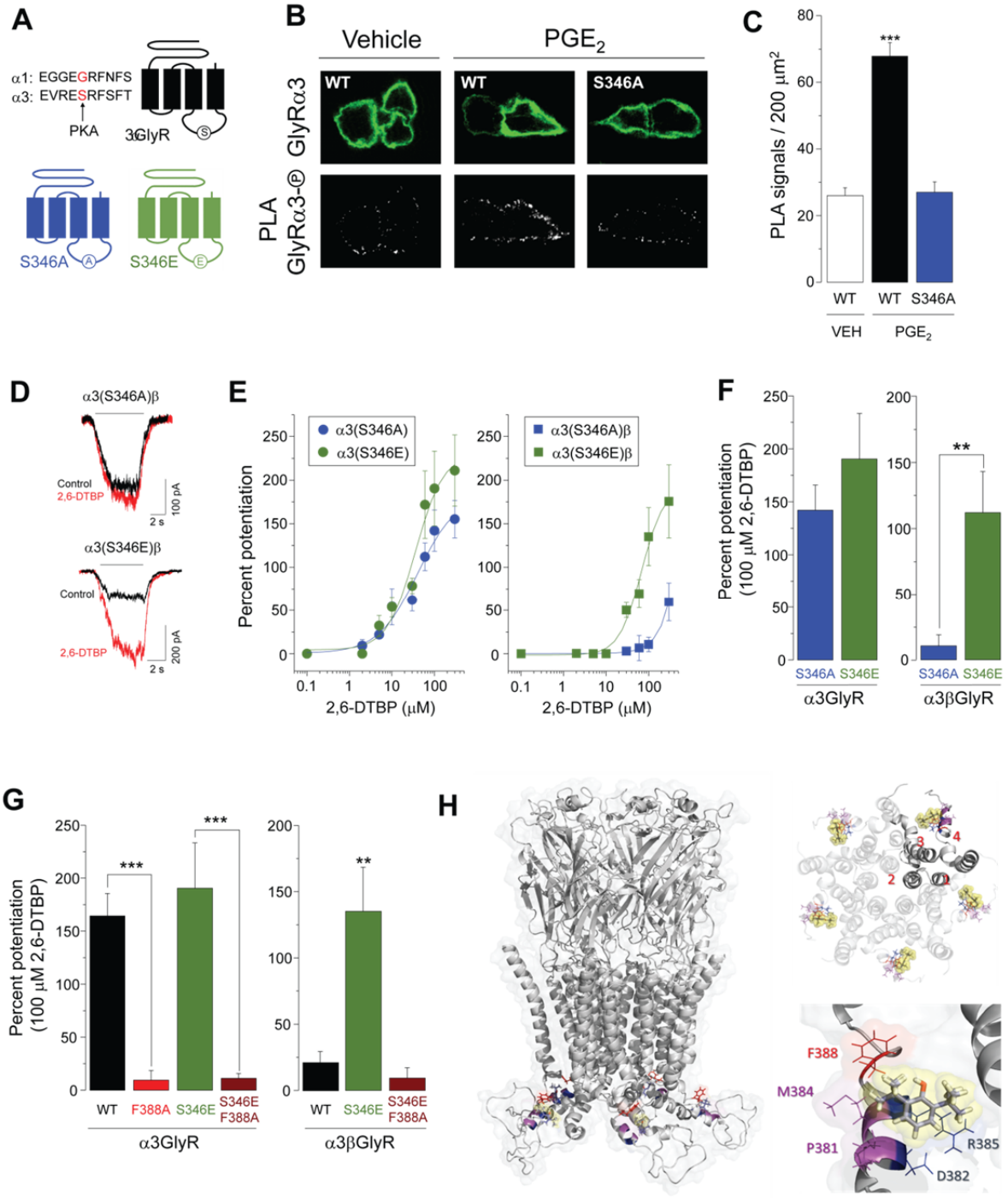


Figure 3. Influence of S346 phosphomutations in α3GlyR on allosteric modulation by 2,6-DTBP. **(A)** Localization of the S346 residue in the primary sequence of the mouse GlyRα3 subunit, corresponding sequence in GlyRα1 subunit, and schematic diagram illustrating this site in the α3GlyR topology. **(B)** Proximity ligation assay (PLA) demonstrating phosphorylation of α3GlyR by PGE_2 (1 µM) in HEK293T cells transfected with EP2 receptor and wild-type or α3(S346A)GlyR subunit plasmids. Top: α3GlyR immunofluorescence, bottom: PLA signal. Scale bar, 5 µm. **(C)** PGE_2 significantly increased PLA signals in HEK293T cells transfected with wild-type but not with (S346A) α3GlyR subunits. ***, $p<0.001$, ANOVA followed by Bonferroni post hoc test, $F(2,141)=49.84$, $p<0.001$, PGE_2 in wild-type α3GlyR transfected cells versus both other conditions. **(D)** Example traces of glycine-activated currents in α3(S346A)β or α3(S346E)β GlyRs in the absence and presence of 2,6-DTBP (100 µM). **(E)** Concentration-response curves to 2,6-DTBP in homomeric α3 (left) and heteromeric α3βGlyRs (right) containing S346E or S346A mutations. **(F)** The phosphomimicking S346E mutation

restored 2,6-DTBP sensitivity of α3β GlyRs but had little effect on that of homomeric α3GlyRs. **, p<0.01, unpaired t-test. **(G)** The F388A point mutation in α3GlyRs abolished 2,6-DTBP sensitivity. Introducing the S346E mutation did not restore 2,6-DTBP sensitivity in homomeric α3(F388A)GlyRs or heteromeric α3(F388A)βGlyRs. **, p<0.01, ***, p<0.001, ANOVA followed by Bonferroni post hoc test, F(3,35)=20.7 (left) and F(2,16)=8.81 (right). **(H)** Structural model of α3GlyR. Left: location of the F388 residue (red) and of 2,6-DTBP (yellow sphere) in a homopentameric α3GlyR. Other relevant residues are shown in magenta (P381 and M384) and blue (D382 and R385). Top right: view from the middle of the plasma membrane on the putative acceptor sites with 2,6-DTBP bound (in yellow spheres) within a pentameric complex. Bottom, right: detailed view of the putative acceptor site of 2,6-DTBP. The hydrophobic interaction supported by F388 is highlighted in red.

Modulation of Gly-IPSCs by 2,6-DTBP in superficial dorsal horn neurons

We next characterized the effects of 2,6-DTBP on native GlyRs in excitatory lamina II dorsal horn neurons, i.e. at a site that serves a pivotal role in pain control and which expresses a high density of α3GlyR (Harvey et al., 2004). To this end, we prepared lumbar spinal cord slices from vGAT::ChR2 BAC transgenic mice (Zhao et al., 2011), which permit selective activation of inhibitory neurons through short pulses of blue light (Foster et al., 2015). In slices taken from these mice, excitatory neurons can be readily identified by the absence of a photocurrent (for details see Methods and (Foster et al., 2015)). Of a total of 256 lamina II dorsal horn neurons, 121 were photocurrent negative and hence presumably excitatory. Wide field stimulation with blue light elicited inhibitory postsynaptic current (IPSC) responses in all these cells. Bicuculline (10 µM) or strychnine (1 µM) were used to isolate glycinergic or GABAergic IPSCs (Gly-IPSCs or GABA-IPSCs), respectively. We then analyzed the effect of 2,6-DTBP (100 µM) on the amplitudes and decay time constants of light-evoked Gly-IPSCs and GABA-IPSCs (Figure 4). As expected from the experiments in recombinant GlyRs, 2,6-DTBP had no significant effect on Gly-IPSCs under basal conditions. Gly-IPSC amplitudes slightly decreased by -8.6 ± 3.1% of control values (n = 8, p = 0.09, paired t-test), while rise time and decay time slightly increased by 13 ± 9% and 14 ± 9% (p = 0.50 and p = 0.17, paired t-test) (Figure 4A-C). Likewise, light-evoked GABA-IPSCs were not significantly affected (-6.1 ± 7.3%; p = 0.57, and 1.1 ± 9.6%; p = 0.92, for amplitudes and decay time constants, n = 6) (Figure 4D-F). It is likely that most of the receptors underlying these synaptic currents were heteromeric receptors containing α1 and/or α3 subunits together with β subunits (Harvey et al., 2004). To address a potential effect on extrasynaptic receptors, we analyzed whether 2,6-DTBP would modulate tonic GlyR currents. In agreement with previous results (Mitchell et al., 2007), these experiments revealed the presence of small amplitude tonic currents (I_{tonic} = 3.2 ± 1.0 pA) in only a small fraction of the superficial dorsal horn neurons (4/14). These tonic currents were not significantly changed by 100 µM 2,6-DTBP (4.6 ± 7.6% of control amplitudes, p = 0.56, paired t-test, Figure S4). The present results indicate that, in slices taken from naïve mice, neither synaptic nor extrasynaptic GlyRs were susceptible to significant modulation by 2,6-DTBP.

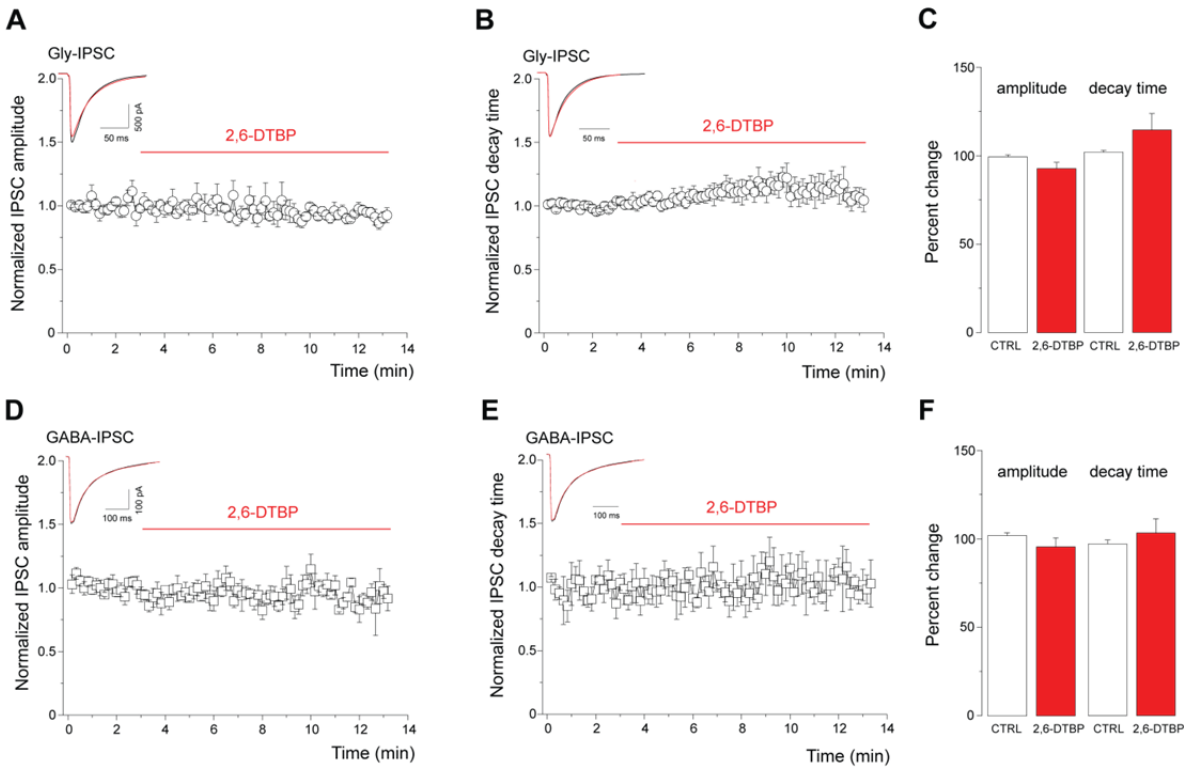


Figure 4. 2,6-DTBP has no significant effects on evoked glycinergic and GABAergic neurotransmission in naïve superficial dorsal horn neurons. (A-C) Effects of 2,6-DTBP (100 µM) on amplitudes (mean ± SEM) (A) or decay time constants (B) of light-evoked Gly-IPSCs. Inset, example glycinergic IPSC traces averaged from 10 consecutive current traces (A) or scales traces (B). Grey, control condition, red 2,6-DTBP. **(C)** Paired t-test, p = 0.12 and p = 0.28, for amplitudes and decay time constants, respectively, n = 6. **(D-F)** Same as (A-C) but GABA-IPSCs. (F) Paired t-test, p = 0.47 and p = 0.37 for amplitudes and decay time constants, respectively, n = 6.

Since our data obtained in recombinant GlyRs have shown that the phosphorylation of the α3 subunit at S346 increases the sensitivity of α3β GlyRs to 2,6-DTBP (compare Figure 3D-F), we hypothesized that glycinergic synapses containing the α3 subunit might be more sensitive to 2,6-DTBP under inflammatory conditions, i.e. in a phosphorylated state. We therefore assessed the effects of 2,6-DTBP on Gly-IPSCs after preconditioning of the slices with PGE_2 (Figure 5). Superfusion of the slices with PGE_2 (1 µM) significantly reduced the amplitude of the Gly-IPSC (-35 ± 5% of control amplitudes, $p < 0.001$, n = 12, paired t-test) but did not change their rise or decay time kinetics (rise time: -0.2 ± 8% change of control, decay time: 6 ± 7% change of control, p = 0.99 and p = 0.23, paired t-test, n = 12) (Figure 5A,D). Subsequent application of 2,6-DTBP significantly increased the decay time constant of Gly-IPSCs by 54 ± 11% ($p < 0.001$, n = 12, paired t-test) but had no significant effect on the amplitudes (+8 ± 10% of amplitudes during PGE_2, p = 0.37, n = 12, paired t-test) (Figure 5A,D). Analyses of the charge transfer occurring during Gly-IPSCs revealed that the prolongation of the decay time by 2,6-DTBP fully compensated for the reduction of Gly-IPSC

amplitudes by PGE_2. The change in IPSC charge transfer during PGE_2 relative to control was -33 ± 5% ($p < 0.001$, paired t-test, n = 12). In the additional presence of 2,6-DTBP, the total charge transfer increased to 108 ± 18% of control (n =12). Superfusion of vehicle (0.1% DMSO) had no effect on either amplitudes or decay kinetics of Gly-IPSCs (8.5 ± 7.5%, p = 0.27, and 19 ± 12%, p = 0.24, for amplitudes and decay time constants, respectively, n = 7). To verify the critical role of PKA-dependent phosphorylation in the priming of spinal cord slices by PGE_2, we pre-incubated slices with the PKA inhibitor H89 (5 µM) (Figure 5B,D). Under these conditions, PGE_2 did no longer sensitize GlyR to the potentiating actions of 2,6-DTBP (change in decay time constant by 2,6-DTBP: -1.8 ± 10.2%, p = 0.73, paired t-test, n = 7). To further prove that GlyRα3 was the relevant target of PKA-dependent phosphorylation, we performed experiments in slices taken from GlyR$\alpha3^{-/-}$ mice (Figure 5C,D). Before that, we tested whether the expression of GlyRα1 or GlyRβ subunits changed following the loss of GlyRα3 expression. Real time PCR analyses of lumber spinal cord dorsal horn tissue from wild-type and GlyR$\alpha3^{-/-}$ mice did not reveal significant changes. mRNA copy numbers relative to β-actin were 0.037 ± 0.017 versus 0.047 ± 0.012 for the α1 subunit (*glra1*; n = 6 for both groups, p = 0.28 unpaired t-test), and 0.021 ± 0.009 versus 0.021 ± 0.004 for the β subunit (*glrb;* n = 6 for both groups, P = 0.99 unpaired t-test) in wild-type versus GlyR$\alpha3^{-/-}$ mice, respectively.

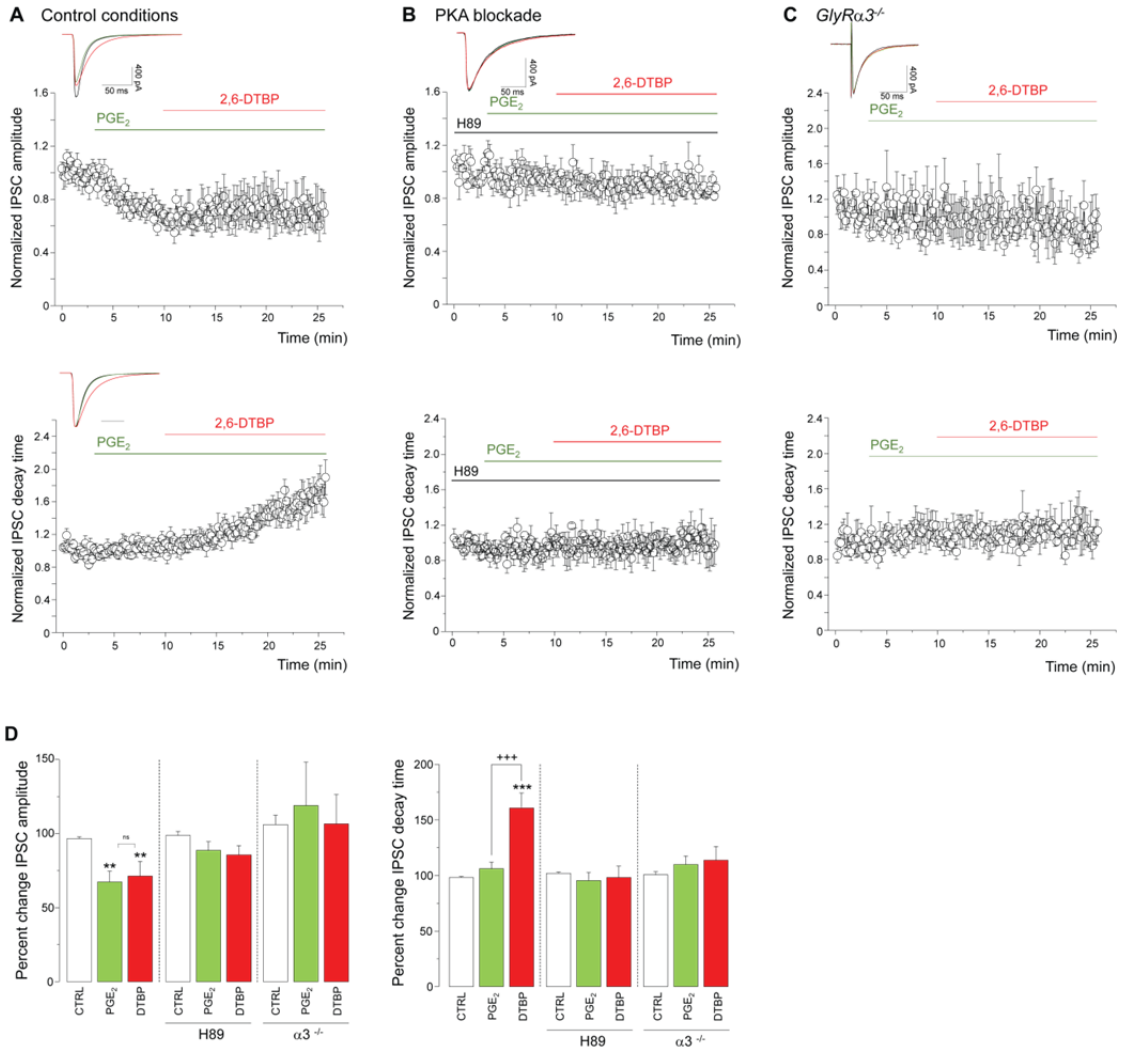


Figure 5. Pretreatment with PGE_2 renders synaptic α3βGlyRs susceptible to modulation by 2,6-DTBP. (A) Normalized amplitudes (mean ± SEM) (top panel) and decay time constants (bottom panel) of light-evoked Gly-IPSC traces versus time, before and during the application of PGE_2 (1 μM) and in the additional presence of 2,6-DTBP (100 μM). Insets are example traces averaged from 10 consecutive stimulations under the three conditions (n = 12). **(B)** Same as (A) but preincubation with H-89 (5 μM) to prevent phosphorylation of GlyRs by PGE_2 (n = 7). **(C)** Same as (A) but experiments done in slices taken from GlyRα3$^{-/-}$ mice. In these experiments, Gly-IPSCs were evoked by electrical field stimulation, n = 12. **(D)** Left panel: PGE_2 reduced Gly-IPSC amplitudes in control slices but not in H89-treated slices or in slices from GlyRα3$^{-/-}$ mice, while 2,6-DTBP had no significant effects on Gly-IPSC amplitudes in either condition. ** $p < 0.01$ significant versus control. ANOVA followed by Bonferroni post hoc test. $F(2,35) = 8.58$, $F(2,18) = 1.76$, and $F(2,30) = 0.12$ for Gly-IPSC amplitudes shown in (A), (B), and (C), respectively. Right panel: 2,6-DTBP significantly prolonged Gly-IPSC decay time courses in PGE_2-treated wild-type slices, but not in slices pre-treated with H89, or in slices prepared from GlyRα3$^{-/-}$ mice. ANOVA followed by Bonferroni post hoc test. $^{+++}$, $p < 0.001$ significant versus PGE_2, *** $p < 0.001$ significant versus control. $F(2,35) = 15.66$, $F(2,18) = 0.20$, and $F(2,30) = 0.62$ for experiments shown in (A), (B), and (C), respectively. n = 7 – 12 cells per group. All scale bars, 50 ms, 400 pA.

These data are consistent with unchanged expression and distribution of the GlyRα1 protein in the dorsal horn of GlyRα3$^{-/-}$ mice reported previously (Figure S1 in ref. 6). Since the vGAT::ChR2 transgene had not been crossed into GlyRα3$^{-/-}$ mice, we evoked glycinergic

IPSCs in these experiments by electrical field stimulation rather than through optogenetic stimulation. In GlyR$\alpha3^{-/-}$ mice, PGE_2 failed to render Gly-IPSCs susceptible to modulation by 2,6-DTBP (change in decay time constant induced by 2,6-DTBP: 1.2 ± 8.1% change of control, p = 0.74, paired t-test, n = 12). Because GlyR$\alpha3^{-/-}$ the mice lacked vGAT::ChR2 transgene, the presence or absence of photocurrents could not be used to distinguish between inhibitory and excitatory neurons. Nevertheless, we expect that at least half of these recordings were made from excitatory neurons (see also ref (Todd & Spike, 1993)). Taken together, both PKA inhibition and absence of GlyR$\alpha3$ prevented the priming effect of PGE_2 on GlyRs supporting that 2,6-DTBP potentiates synaptic GlyRs in a phosphorylation state-dependent dependent manner.

In order to show that this priming also occurred *in vivo* in response to peripheral inflammation, we studied the effects of 2,6-DTBP on Gly-IPSCs in slices prepared from five mice with an inflamed hindpaw (Figure 6). Inflammation was induced by subcutaneous injection of zymosan A into the left hindpaw (Meller & Gebhart, 1997) and the development of inflammatory hyperalgesia was confirmed by monitoring mechanical response thresholds. Application of 2,6-DTBP did not significantly alter the Gly-IPSC amplitudes (+3.6 ± 9.7% of control amplitudes, p = 0.72, paired t-test) or rise times (+13.5 ± 3.8%, p = 0.09, paired t-test). However, it significantly increased decay time kinetics by 23.5 ± 7.5% (p < 0.01, paired t-test). Application of vehicle instead of 2,6-DTBP did not significantly change Gly-IPSC amplitudes or decay time constants (data not shown).

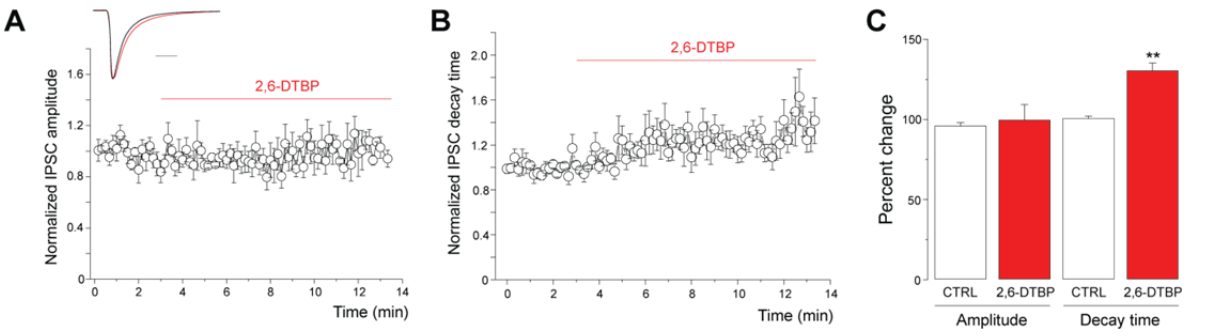


Figure 6. Effects of 2,6-DTBP on the amplitude and decay time of Gly-IPSCs recorded from lamina II neurons of mice with inflamed hindpaws. Recordings were made from the dorsal horn neurons ipsilateral to the inflamed paw. (**A**) Normalized Gly-IPSC amplitudes over time of whole cell recording. (**B**) Same as (A) but normalized decay time constant. (**C**) 2,6-DTBP had no significant effect on IPSC amplitudes, but significantly increased the decay time constant. **, p < 0.01, paired t-test, n = 6 cells.

Anti-hyperalgesic activity of 2,6-DTBP in behavioral models of pain

Finally, we investigated whether the modulatory effects of 2,6-DTBP on GlyRs translate to antihyperalgesic activity in inflammatory pain *in vivo*. To this end, we again induced

inflammatory hyperalgesia in mice through subcutaneous injection of zymosan A and tested the effects of 2,6-DTBP on mechanical and heat hyperalgesia 48 hours after zymosan A injection (i.e. when central sensitization had reached its maximum; (Reinold et al., 2005)). 2,6-DTBP significantly reduced mechanical and heat hyperalgesia with a maximum response occurring 30 - 60 min after drug administration (Figure 7A,B). 2,6-DTBP reversed mechanical and heat hyperalgesia at all the time points examined (two-way repeated measures ANOVA followed, $p < 0.05$), whereas vehicle did not elicit significant effects. Consistent with our finding that phosphorylation was required to render GlyRs sensitive to 2,6-DTBP, we found that 2,6-DTBP had no analgesic effects against acute nociceptive stimuli (pin-prick) in naïve mice (withdrawal responses changes from 89 ± 6% to 92 ± 3% 30 - 90 min after 2,6-DTBP, $p = 0.48$, paired t-test, $n = 6$) (Figure S5A). To exclude possible confounding effects of 2,6-DTBP on locomotor activity, motor coordination and muscle strength we tested the effects of 2,6-DTBP on locomotion in an open field arena, in the accelerating rotarod test and in the horizontal wire test. No significant effects were observed (Figure S5B-D).

To determine the contribution of α3GlyRs to 2,6-DTBP-induced analgesia, we compared the antihyperalgesic effects of 2,6-DTBP in wild-type and GlyR$\alpha3^{-/-}$ mice in different pain models (Figure 7C,D). We first address inflammatory hyperalgesia. Because previous studies have shown that GlyR$\alpha3^{-/-}$ mice recover quickly from zymosan A-induced hypersensitivity (Harvey et al., 2004), we switched to Freund's adjuvant (CFA), which causes more prolonged hyperalgesia, and tested the effects of 2,6-DTBP at an early time point (48 hrs) after CFA injection. Wild-type and GlyR$\alpha3^{-/-}$ mice did not significantly differ in their mechanical paw withdrawal thresholds (PWT) at this time point and at baseline (Figure 7C). However, heat hyperalgesia in inflamed GlyR$\alpha3^{-/-}$ mice was much less pronounced than in wild-type mice precluding a quantitative comparison of antihyperalgesia by 2,6-DTBP in inflamed wild-type and GlyR$\alpha3^{-/-}$ mice (Figure S6). We therefore focused on mechanical hyperalgesia, which is also more relevant to chronic pain in human patients (Schaible 2013). 2,6-DTBP significantly reduced mechanical hyperalgesia in wild-type mice by 44.3 ± 5.2% (Figure 7C). In GlyR$\alpha3^{-/-}$ mice, this effect was reduced to 16.0 ± 6.2%, indicating that about two thirds of the 2,6-DTBP-evoked analgesia came from an interaction with α3GlyRs. Previous work from our group suggested that phosphorylation and inhibition of GlyRα3 contributes to inflammatory but not to neuropathic hyperalgesia (Hösl et al., 2006), while others have shown that 2,6-DTBP still alleviated neuropathic hyperalgesia (Tibbs et al., 2013). We therefore investigated whether the antihyperalgesic effects of 2,6-DTBP in neuropathic pain also depended on GlyRα3. To this end, we examined the effects of 2,6-DTBP on neuropathic hyperalgesia in wild-type and GlyR$\alpha3^{-/-}$ mice. Neuropathic hyperalgesia was induced applying a chronic constriction injury of the left sciatic nerve (CCI, (Bennet & Xie, 1988)). 2,6-DTBP reduced

hyperalgesia in both wild-type and GlyRα3$^{-/-}$ mice to very similar degrees (Figure 7D, see also Figure S7). The maximum antihyperalgesic responses, which were reached between 30 and 60 min after the drug administration, were not significantly different between wild-type and GlyRα3$^{-/-}$ mice (wild-type: 46.1 ± 4.6% versus GlyRα3$^{-/-}$: 41.0 ± 4.7% of maximum possible effect, MPE), indicating that antihyperalgesic actions against neuropathic pain occurred independent of GlyRα3. Taken together, these findings indicate that dorsal horn GlyRs are modulated by 2,6-DTBP in a phosphorylation state-dependent manner and that this modulation is particularly relevant for inflammatory hyperalgesia. 2,6-DTBP also exerts antihyperalgesia against neuropathic pain, but these actions occurred through mechanisms different from α3GlyRs possibly involving α1GlyRs (Ahrens et al., 2004) or HCN channels (Tibbs et al., 2013).

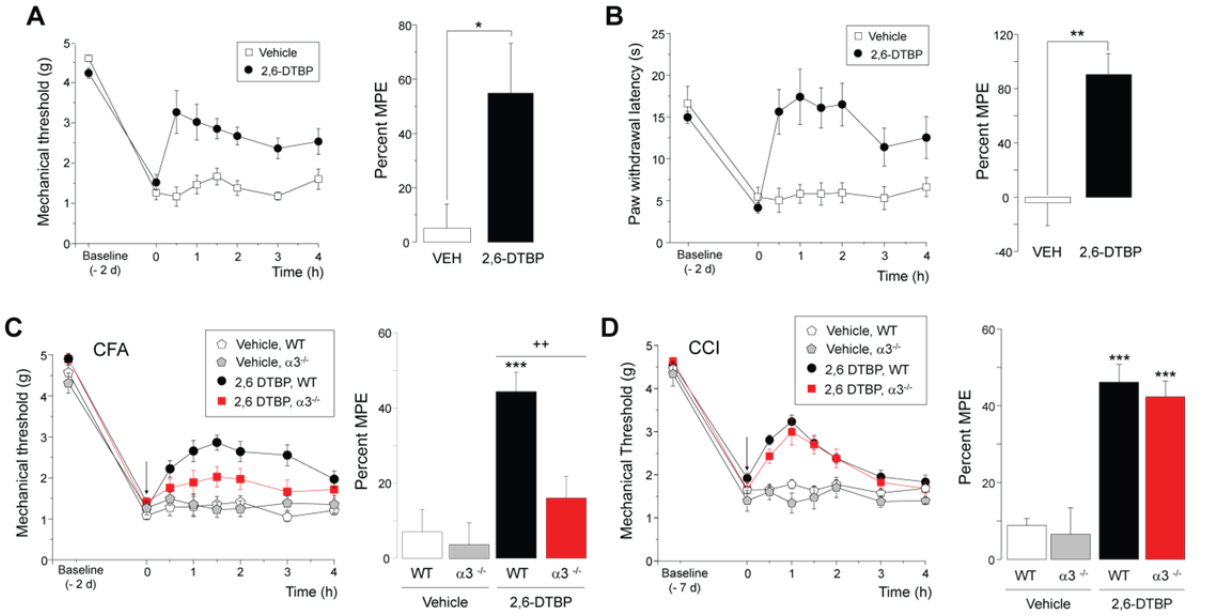


Figure 7. *In vivo* antihyperalgesic effects of 2,6-DTBP. (A,B) Zymosan A model of inflammatory hyperalgesia. Zymosan A (0.06 mg) was injected subcutaneously into one hindpaw. 2,6-DTBP (90 mg/kg i.p.) or vehicle were given 48 hours after zymosan A. (**A**) Mechanical hyperalgesia. Treatment*time interactions ($p<0.05$) were significant for time points between 30 min and 2 hrs (two-way repeated measures ANOVA, $F(1,6)=5.0–8.2$). Right: maximum possible antihyperalgesic effects (MPE) determined for the interval 30-60 min after 2,6-DTBP injection. *, $p<0.05$, unpaired t-test, n=6 and 7 mice for 2,6-DTPB and vehicle, respectively. **(B)** Same as (A), but heat hyperalgesia. Treatment*time interactions were significant for time points between 30 min and 4 hrs ($F(1,6)=7.2–24.8$). MPEs: Unpaired t-test, **, $p<0.01$, n=7 and 6. **(C)** Inflammatory hyperalgesia evoked by CFA. 2,6-DTBP was applied 2 days after CFA injection. In wild-type mice (n=7 and 9), treatment*time interactions were significant ($p<0.05$) for time points between 30 min and 4 hrs ($F(1,6)=4.8–20.0$). In GlyRα3$^{-/-}$ mice (n=10 and 7), interactions remained insignificant for all time points ($p>0.17$; $F(1,6)=0.02–0.7$). Significant MPE of 2,6-DTBP (determined between 60-90 min) were found in wild-type, but not in GlyRα3$^{-/-}$ mice. MPE differed significantly between wild-type and GlyRα3$^{-/-}$ mice (ANOVA followed by Bonferroni post hoc test, $F(3,38)=9.97$; ***, $p<0.001$, for vehicle versus 2,6-DTBP in wild-type mice); $^{++}$, $p<0.01$ (2,6-DTBP effect in wild-type versus GlyRα3$^{-/-}$ mice). **(D)** Neuropathic hyperalgesia. 2,6-DTBP was applied 7 days after CCI. In wild-type mice, treatment*time interactions were significant ($p<0.05$) between 30 min and 90 min ($F(1,6)=0.09–69.0$). In GlyRα3$^{-/-}$ mice, a

significant interaction ($p<0.05$) was found only for 60 min ($F(1,6)=0.003–5.9$). MPE was significant in wild-type and GlyR$\alpha3^{-/-}$ mice (ANOVA followed by Bonferroni post-hoc test, $F(3,30)=24.7$; ***, $p<0.001$, for 2,6-DTBP versus vehicle). No significant difference was found between 2,6-DTBP treated wild-type and GlyR$\alpha3^{-/-}$ mice ($p=0.55$).

DISCUSSION

Our results establish the non-anesthetic propofol derivative 2,6-DTBP as an efficacious enhancer of glycinergic inhibition in the superficial spinal dorsal horn, a key area for central pain control. 2,6-DTBP not only spared $GABA_A$Rs, but also was particularly effective at α3 containing GlyRs, which are abundant in the spinal termination area of nociceptive nerve fibers. While homomeric α3GlyRs were potentiated by 2,6-DTBP under basal conditions, heteromeric (β subunit containing) GlyRs, which prevail at postsynaptic sites, were potentiated only in a phosphorylated ("primed") state. In line with these results, 2,6-DTBP potentiated synaptic GlyR receptor currents in spinal cord slices only after priming with the inflammatory mediator PGE_2 or in slices that were obtained from mice with peripheral inflammation. The phosphorylation site responsible for this priming effect is present in α3GlyRs but absent in α1GlyRs.

Mechanisms and sites for modulation

When homomeric GlyR channels expressed in HEK293T cells were investigated, 2,6-DTBP shifted the glycine concentration response curve to the left. This leftward shift corresponds well with the increase in channel open probability observed in our single channel recording experiments. In spinal cord slices primed with PGE_2, 2,6-DTBP prolonged the decay of synaptic GlyR currents without altering their amplitudes consistent with the observed shift in the glycine concentration response curve. In terms of gross glycinergic inhibition (measured as total charge transfer), this prolongation of the decay time course at least partially counteracted the reduction in the amplitude of glycinergic responses seen after PGE_2.

Our present results indicate that modulation of α3GlyRs by 2,6-DTBP depends on a single phenylalanine residue (F388), which is also required for the modulation of homomeric glycine receptors by propofol, the parent compound of 2,6-DTBP (Moraga-Cid et al., 2011). Up to now, a potential role in this process of the β subunit and of posttranslational modifications has remained unclear. In the present study we show that heteromeric α3βGlyRs (in their non-phosphorylated state) are much less susceptible to modulation by 2,6-DTBP. This is consistent with the presence of an isoleucine residue instead of phenylalanine at the respective position in the β subunit (I443) and may in addition suggest that the presence of β subunit prevents 2,6-DTBP from accessing its binding site in the α subunit (compare Figure S3). Crystal structures for TM3-TM4 intracellular regions and for heteromeric Cys-loop ion channels are still lacking. However, our homology modeling data (Figure 3H) suggests that F388 lies in the so called membrane associated (MA) stretch, i.e. in a region that can influence some biophysical properties of ion channels (Carland et al., 2009). A comparison of the models of the α3-α3-α3 and α3-β-α3 interfaces suggests that the volume of the β subunit

MA-stretch is considerably larger than that of the α3 subunit (by approximately 14500 Å^3; Figure S8). Together with the functional data, these analyses suggest that, in homomeric α3 GlyRs, all five potential binding sites are accessible to 2,6-DTBP, whereas the presence of β subunits in the receptor complex decreases not only the number of acceptor sites but also renders the acceptor sites within the α subunits less accessible to 2,6-DTBP (at least as long the receptors are in the non-phosphorylated state). An unexpected finding was that the susceptibility of modulation by 2,6-DTBP of heteromeric α3βGlyRs was restored by phosphorylation of (or introduction of phosphomimetic mutations in) the α3 subunit at S346. This observation suggests a phosphorylation-induced conformational change on the α3 subunit permitting modulation of α3βGlyRs by 2,6-DTBP (Figure S9).

Does modulation of GlyRs contribute to analgesic actions of 2,6-DTBP?

2,6-DTBP does not only potentiate GlyRs but also inhibits hyperpolarization and cyclic nucleotide gated (HCN) ion channels (Tibbs et al., 2013), in particular HCN1 channels, which are expressed in peripheral nociceptors and contribute to pain sensitization (Momin et al., 2008). In addition, antioxidant properties of 2,6-DTBP (Ku et al., 1990; Shakir et al., 2014) may directly or indirectly (e.g. via inhibition of T-type Ca^{2+} channel, ref (Nelson et al., 2007)) contribute to antihyperalgesia. Our finding that the analgesic effects of 2,6-DTBP in mice with inflamed paws were reduced by about two-thirds in GlyRα3$^{-/-}$ mice indicates that α3GlyRs contribute significantly to the analgesic action of 2,6-DTBP. In line with a previous report (Tibbs et al., 2013), we found that 2,6-DTBP was active not only against inflammatory pain but also against neuropathic pain caused by peripheral nerve damage. Analgesia against the latter was however not diminished in GlyRα3$^{-/-}$ mice consistent with our previous data that suggested that phosphorylation of α3GlyRs does not contribute to neuropathic hyperalgesia (Hösl et al., 2006).

In addition to strychnine-sensitive GlyRs, spinal $GABA_A$Rs constitute another target that might allow restoring proper synaptic inhibition in the spinal cord during chronic pain states. $GABA_A$Rs offer plenty of opportunities for pharmacological interventions, but currently available drugs targeting $GABA_A$Rs do not exert clinically relevant analgesia mainly because of dose limiting sedative effects (Ralvenius et al., 2015). Targeting GlyRs offers the advantage that glycinergic innervation is largely restricted to the hindbrain and spinal cord, and almost completely spares the forebrain, where most of the unwanted actions of GABAergic drugs originate. Consistent with this concept, 2,6-DTBP apparently lacks effects on locomotor activity, motor coordination and muscle strength, which are typical side effects of classical GABAergic drugs such as the benzodiazepines. It is likely that both the low abundance and absence of glycinergic innervation in the forebrain and specific modulation of

phosphorylated GlyRs contribute to this favorable profile.

2,6-DTBP did not only show a preference for GlyRs over $GABA_A$Rs but also preferred the low abundance α3GlyRs over the much more prevalent α1GlyRs; the other two GlyRs isoforms (α2 and α4GlyRs) are less relevant as drug targets as α2GlyRs are mainly expressed during prenatal and early postnatal development (Lynch, 2009), while the gene encoding the α4 subunit is a pseudogene in humans (Laube et al., 2002; Simon et al., 2004). Compared to α1GlyRs, which are found throughout most parts of the spinal cord and hindbrain, α3GlyRs are expressed in a spatially much more restricted manner. In the spinal cord, α3GlyR expression is limited to the superficial layers of the dorsal horn (Harvey et al., 2004), while at supra-spinal sites, α3GlyR expression is generally weak and found only in a few sites, such the retina (Haverkamp et al., 2003), cerebellum and the hippocampus (Malosio et al., 1991). The preferential potentiation of α3GlyRs may thus help avoiding unwanted effects such as strong muscle relaxation.

An additional level of specificity comes from the dependence of modulation of synaptic GlyRs on prior phosphorylation. Most postsynaptic GlyRs are heteromers containing in addition to α subunits also β subunits, which anchor the channel complexes to postsynaptic sites. Our experiments revealed that, under resting (non-phosphorylated) conditions, heteromeric α3βGlyRs were much less sensitive to modulation by 2,6-DTBP than homomeric receptors. However, introduction of a phosphomimetic amino acid exchange in the α3 subunit of recombinant α3βGlyRs restored susceptibility to modulation by 2,6-DTBP. Accordingly, native postsynaptic GlyRs of the spinal dorsal horn were only modulated after priming through pretreatment of spinal cord slices with PGE_2, which leads to PKA-dependent phosphorylation of α3GlyRs, or, when slices were taken from mice with an inflamed hindpaw. Importantly, the consensus site for PKA-dependent phosphorylation of GlyR α3 subunits is missing from the α1 subunit introducing again an additional level of specificity. The absence of this site in GlyR α1 subunits may explain why we did not find effects of 2,6-DTBP on muscle strength although GlyRs effectively control motoneuron excitability.

Recordings of synaptic GlyR currents in slices obtained from naïve (non-inflamed) mice were not modulated by 2,6-DTBP suggesting that homomeric α3GlyRs lacking GlyR β subunits do not make a measureable contribution to synaptic inhibition in the dorsal horn. However, homomeric receptors may be present at extrasynaptic sites where they mediate tonic currents. In line with a previous report (Mitchell et al., 2007) we found tonic currents only in a small portion of cells and these currents were of very small (≤ 5 pA) amplitudes. In certain areas of the brainstem, homomeric α1GlyRs have been reported to be present in presynaptic terminals where they control transmitter release (Xiong et al., 2014; Hruskova et al., 2012).

The lack of modulation of glycinergic synaptic currents by 2,6-DTBP suggests that such homomeric presynaptic GlyRs are not present in the superficial dorsal horn. It is however possible that such receptors exist at other sites and that interaction with these receptors might cause effects of 2,6-DTBP on behaviors of mice not studied here.

In summary, our results describe a phosphorylation state-dependent interaction of a propofol derivative with inflammation-primed synaptic GlyRs in the spinal dorsal horn. Reduced analgesic effects in GlyRα3-deficient mice suggest that this phosphorylation-dependent potentiation contributes to the analgesic effects of 2,6-DTBP against inflammatory pain (Figure S10). These results provide direct evidence that diminished inhibitory pain control in the spinal cord can be restored by positive allosteric modulation of GlyRs and may pave the path for the generation of new spinally-acting analgesics with limited supraspinal side effects.

ACKNOWLEDGMENTS

We are thankful to Thomas Grampp and Louis Scheurer for excellent technical assistance, to Isabelle Kellenberger for mouse genotyping, and to Carmen Brazerol, Conny Leuzinger, Dennis Kwame and Ana Hemmersbach for animal care taking. This work was supported by grants from the Swiss National Science Foundation (SNSF; 131093) and an advanced investigator grant from the European Research Council (ERC AdG DHISP 250128) to H.U.Z., by grants from the Fondation de la Recherche Médicale, Institut Pasteur, to G.M.-C. and P.J.C. and the Agence Nationale de la Recherche Grants to P.J.C., VRID-University of Concepcion 213.033.106-1.0, and FONDECYT 1140515 to G.E.Y. G.E.Y. was supported through a Forschungskredit from the University of Zurich.

REFERENCES

Basbaum AI, Bautista DM, Scherrer G, and Julius D. Cellular and molecular mechanisms of pain. *Cell.* 2009;139(2):267-84.

Zeilhofer HU, Benke D, and Yévenes GE. Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control. *Annu Rev Pharmacol Toxicol.* 2012;52(111-33.

Luo C, Kuner T, and Kuner R. Synaptic plasticity in pathological pain. *Trends Neurosci.* 2014;37(6):343-55.

Sandkühler J. Models and mechanisms of hyperalgesia and allodynia. *Physiol Rev.* 2009;89(2):707-58.

Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De Koninck P, and De Koninck Y. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature.* 2003;424(6951):938-42.

Harvey RJ, Depner UB, Wässle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schutz B, Abo-Salem OM, et al. GlyRα3: an essential target for spinal PGE_2-mediated inflammatory pain sensitization. *Science.* 2004;304(5672):884-7.

Lynch JW. Native glycine receptor subtypes and their physiological roles. *Neuropharmacology.* 2009;56(1):303-9.

Yévenes GE, and Zeilhofer HU. Allosteric modulation of glycine receptors. *Br J Pharmacol.* 2011a;164(2):224-36.

Laube B, Maksay G, Schemm R, and Betz H. Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? *Trends Pharmacol Sci.* 2002;23(11):519-27.

de la Roche J, Leuwer M, Krampfl K, Haeseler G, Dengler R, Buchholz V, and Ahrens J. 4-Chloropropofol enhances chloride currents in human hyperekplexic and artificial mutated glycine receptors. *BMC neurology.* 2012;12,104.

Eckle VS, Grasshoff C, Mirakaj V, O'Neill PM, Berry NG, Leuwer M, and Antkowiak B. 4-bromopropofol decreases action potential generation in spinal neurons by inducing a glycine receptor-mediated tonic conductance. *Br J Pharmacol.* 2014;171(24):5790-801.

Xiong W, Cheng K, Cui T, Godlewski G, Rice KC, Xu Y, and Zhang L. Cannabinoid potentiation of glycine receptors contributes to cannabis-induced analgesia. *Nat Chem Biol.* 2011;7:296-303.

Xiong W, Cui T, Cheng K, Yang F, Chen SR, Willenbring D, Guan Y, Pan HL, Ren K, Xu Y, et al. Cannabinoids suppress inflammatory and neuropathic pain by targeting α3 glycine receptors. *J Exp Med.* 2012;209(6):1121-34.

Yang Z, Aubrey KR, Alroy I, Harvey RJ, Vandenberg RJ, Lynch JW. Subunit-specific modulation of glycine receptors by cabnnabinoids and N-arachidonyl-glycine. *Biochem Pharmacol.* 2008;76(8):1014-1023

Zhang JY, Gong N, Huang JL, Guo LC, and Wang YX. Gelsemine, a principal alkaloid from Gelsemium sempervirens Ait., exhibits potent and specific antinociception in chronic pain by acting at spinal α3 glycine receptors. *Pain.* 2013;154(11):2452-62.

Ahrens J, Haeseler G, Leuwer M, Mohammadi B, Krampfl K, Dengler R, and Bufler J. 2,6 di-tert-butylphenol, a nonanesthetic propofol analog, modulates $\alpha1\beta$ glycine receptor function in a manner distinct from propofol. *Anesthesia and analgesia.* 2004;99(1):91-6.

Ahrens J, Leuwer M, de la Roche J, Foadi N, Krampfl K, and Haeseler G. The non-anaesthetic propofol analogue 2,6-di-tert-butylphenol fails to modulate $GABA_A$ receptor function. *Pharmacology.* 2009;83(2):95-8.

Krasowski MD, Jenkins A, Flood P, Kung AY, Hopfinger AJ, and Harrison NL. General anesthetic potencies of a series of propofol analogs correlate with potency for potentiation of γ-aminobutyric acid (GABA) current at the $GABA_A$ receptor but not with lipid solubility. *J Pharmacol Exp Ther.* 2001;297(1):338-51.

Marabelli A, Moroni M, Lape R, and Sivilotti LG. The kinetic properties of the α3 rat glycine receptor make it suitable for mediating fast synaptic inhibition. *The Journal of physiology.* 2013;591(Pt 13):3289-308.

Ahrens J, Leuwer M, Stachura S, Krampfl K, Belelli D, Lambert JJ, and Haeseler G. A transmembrane residue influences the interaction of propofol with the strychnine-sensitive glycine α1 and α1β receptor. *Anesth Analg.* 2008;107(6):1875-83.

Moraga-Cid G, Yevenes GE, Schmalzing G, Peoples RW, and Aguayo LG. A Single phenylalanine residue in the main intracellular loop of α1 γ-aminobutyric acid type A and glycine receptors influences their sensitivity to propofol. *Anesthesiology.* 2011;115(3):464-73.

Lynagh T, and Laube B. Opposing effects of the anesthetic propofol at pentameric ligand-gated ion channels mediated by a common site. *J Neurosci.* 2014;34(6):2155-9.

Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, et al. Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. *Nature.* 1997;389(6649):385-9.

Findlay GS, Phelan R, Roberts MT, Homanics GE, Bergeson SE, Lopreato GF, Mihic SJ, Blednov YA, and Harris RA. Glycine receptor knock-in mice and hyperekplexia-like phenotypes: comparisons with the null mutant. *J Neurosci.* 2003;23(22):8051-9.

Carland JE, Cooper MA, Sugiharto S, Jeong HJ, Lewis TM, Barry PH, Peters JA, Lambert JJ, and Moorhouse AJ. Characterization of the effects of charged residues in the intracellular loop on ion permeation in α1 glycine receptor channels. *J Biol Chem.* 2009;284(4):2023-30.

Leuchowius KJ, Jarvius M, Wickstrom M, Rickardson L, Landegren U, Larsson R, Soderberg O, Fryknas M, and Jarvius J. High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol Cell Proteomics.*2010.9(1):178-83.

Hibbs RE, and Gouaux E. Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature.* 2011;474(7349):54-60.

Moraga-Cid G, Sauguet L, Huon C, Malherbe L, Girard-Blanc C, Petres S, Murail S, Taly A, Baaden M, Delarue M, et al. Allosteric and hyperekplexic mutant phenotypes investigated on an α1 glycine receptor transmembrane structure. *Proc Natl Acad Sci U S A.* 2015;112(9):2865-70.

Zhao S, Ting JT, Atallah HE, Qiu L, Tan J, Gloss B, Augustine GJ, Deisseroth K, Luo M, Graybiel AM, et al. Cell type-specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function. *Nat Methods.* 2011;8(9):745-52.

Foster E, Wildner H, Tudeau L, Haueter S, Ralvenius WT, Jegen M, Johannssen H, Hosli L, Haenraets K, Ghanem A, et al. Targeted ablation, silencing, and activation establish glycinergic dorsal horn neurons as key components of a spinal gate for pain and itch. *Neuron.* 2015;85(6):1289-304.

Mitchell EA, Gentet LJ, Dempster J, and Belelli D. $GABA_A$ and glycine receptor-mediated transmission in rat lamina II neurones: relevance to the analgesic actions of neuroactive steroids. *J Physiol.* 2007;583(Pt 3):1021-40.

Todd AJ, and Spike RC. The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. *Prog Neurobiol.* 1993;41(5):609-45.

Meller ST, and Gebhart GF. Intraplantar zymosan as a reliable, quantifiable model of thermal and mechanical hyperalgesia in the rat. *Eur J Pain.* 1997;1(1):43-52.

Reinold H, Ahmadi S, Depner UB, Layh B, Heindl C, Hamza M, Pahl A, Brune K, Narumiya S, Müller U, et al. Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. *J Clin Invest.* 2005;115(3):673-9.

Schaible H-G. In: McMahon SB, Koltzenburg M, Tracey I, and Turk DC eds. *Wall and Melzack's Textbook of Pain*. Philadelphia: Elsevier; 2013:609-19.

Hösl K, Reinold H, Harvey RJ, Müller U, Narumiya S, and Zeilhofer HU. Spinal prostaglandin E receptors of the EP2 subtype and the glycine receptor α3 subunit, which mediate

central inflammatory hyperalgesia, do not contribute to pain after peripheral nerve injury or formalin injection. *Pain.* 2006;126(46-53.

Tibbs GR, Rowley TJ, Sanford RL, Herold KF, Proekt A, Hemmings HC, Jr., Andersen OS, Goldstein PA, and Flood PD. HCN1 channels as targets for anesthetic and nonanesthetic propofol analogs in the amelioration of mechanical and thermal hyperalgesia in a mouse model of neuropathic pain. *J Pharmacol Exp Ther.* 2013;345(3):363-73.

Bennett GJ, and Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain.* 1988;33(1):87-107.

Momin A, Cadiou H, Mason A, and McNaughton PA. Role of the hyperpolarization-activated current Ih in somatosensory neurons. *J Physiol.* 2008;586(Pt 24):5911-29.

Ku G, Doherty NS, Schmidt LF, Jackson RL, and Dinerstein RJ. Ex vivo lipopolysaccharide-induced interleukin-1 secretion from murine peritoneal macrophages inhibited by probucol, a hypocholesterolemic agent with antioxidant properties. *FASEB J.* 1990;4(6):1645-53.

Shakir RM, Ariffin A, and Abdulla MA. Synthesis of new 2,5-di-substituted 1,3,4-oxadiazoles bearing 2,6-di-tert-butylphenol moieties and evaluation of their antioxidant activity. *Molecules.* 2014;19(3):3436-49.

Nelson MT, Joksovic PM, Su P, Kang HW, Van Deusen A, Baumgart JP, David LS, Snutch TP, Barrett PQ, Lee JH, et al. Molecular mechanisms of subtype-specific inhibition of neuronal T-type calcium channels by ascorbate. *J Neurosci.* 2007;27(46):12577-83.

Ralvenius WT, Benke D, Acuña MA, Rudolph U, and Zeilhofer HU. Analgesia and Unwanted Benzodiazepine Effects in Point-Mutated Mice Expressing Only One Benzodiazepine-Sensitive $GABA_A$ Receptor Subtype. *Nature Communications.* 2015;6:6803.

Simon J, Wakimoto H, Fujita N, Lalande M, and Barnard EA. Analysis of the set of $GABA_A$ receptor genes in the human genome. *J Biol Chem.* 2004;279(40):41422-35.

Haverkamp S, Müller U, Harvey K, Harvey RJ, Betz H, and Wässle H. Diversity of glycine receptors in the mouse retina: localization of the α3 subunit. *J Comp Neurol.* 2003;465(4):524-39.

Malosio ML, Marqueze-Pouey B, Kuhse J, and Betz H. Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *Embo J.* 1991;10(9):2401-9.

Xiong W, Chen SR, He L, Cheng K, Zhao YL, Chen H, Li DP, Homanics GE, Peever J, Rice KC, et al. Presynaptic glycine receptors as a potential therapeutic target for hyperekplexia disease. *Nat Neurosci.* 2014;17(2):232-9.

Hruskova B, Trojanova J, Kulik A, Kralikova M, Pysanenko K, Bures Z, Syka J, Trussell LO, and Turecek R. Differential distribution of glycine receptor subtypes at the rat calyx of Held synapse. *J Neurosci.* 2012;32(47):17012-24.

Yévenes GE, and Zeilhofer HU. Molecular sites for the positive allosteric modulation of glycine receptors by endocannabinoids. *PLoS ONE.* 2011b;6(8):e23886.

Burgos CF, Castro PA, Mariqueo T, Bunster M, Guzman L, and Aguayo LG. Evidence for α-helices in the large intracellular domain mediating modulation of the α1-glycine receptor by ethanol and G$\beta\gamma$. *J Pharmacol Exp Ther.* 2015;352(1):148-55.

Xu D, and Zhang Y. Ab initio protein structure assembly using continuous structure fragments and optimized knowledge-based force field. *Proteins.* 2012;80(7):1715-35.

Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, Pieper U, and Sali A. Comparative protein structure modeling using Modeller. *Curr Protoc Bioinformatics.* 2006;Chapter 5.

Hassaine G, Deluz C, Grasso L, Wyss R, Tol MB, Hovius R, Graff A, Stahlberg H, Tomizaki T, Desmyter A, et al. X-ray structure of the mouse serotonin 5-HT3 receptor. *Nature.* 2014;512(7514):276-81.

Irwin JJ, Sterling T, Mysinger MM, Bolstad ES, and Coleman, RG 2012. ZINC: a free tool to discover chemistry for biology. *J Chem Inf Model* 2012;52(7):1757-68.

Friesner RA, Murphy RB, Repasky MP, Frye LL, Greenwood JR, Halgren TA, Sanschagrin PC, and Mainz DT. Extra precision glide: docking and scoring incorporating a model

of hydrophobic enclosure for protein-ligand complexes. *J Med Chem.* 2006;49(21):6177-96.

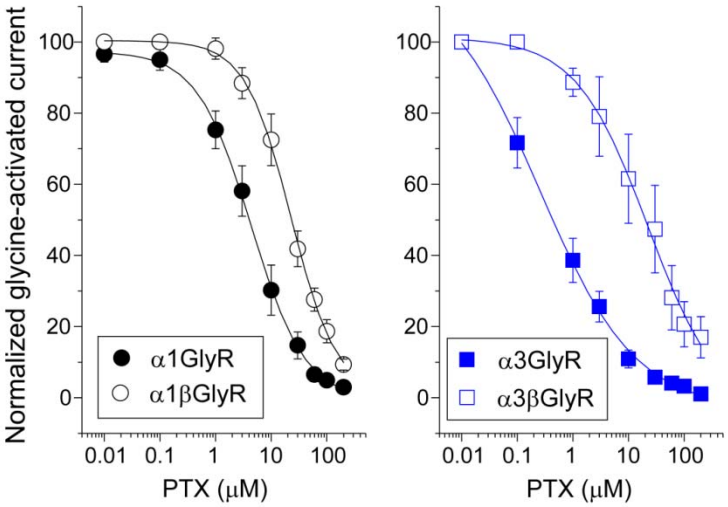
Mowrey DD, Cui T, Jia Y, Ma D, Makhov AM, Zhang P, Tang P, and Xu Y. Open-channel structures of the human glycine receptor α1 full-length transmembrane domain. *Structure.* 2013;21(10):1897-904.

Zemoura K, and Benke D. Proteasomal degradation of γ-aminobutyric acidB receptors is mediated by the interaction of the $GABA_{B2}$ C terminus with the proteasomal ATPase Rtp6 and regulated by neuronal activity. *J Biol Chem.* 2014.289(11):7738-46.

James R, and Glen JB. Synthesis, biological evaluation, and preliminary structure-activity considerations of a series of alkylphenols as intravenous anesthetic agents. *J Med Chem.* 1980;23(12):1350-7.

Bonetti EP, Pieri L, Cumin R, Schaffner R, Pieri M, Gamzu ER, Muller RK, and Haefely W. Benzodiazepine antagonist Ro 15-1788: neurological and behavioral effects. *Psychopharmacology (Berl).* 1982;78(1):8-18.

Supplementary Figures and Tables



GlyR	IC_{50} (μM)	n_H
α1	4.4±0.2	0.8±0.06
α1β	22.7±1.7*	1.06±0.06
α3	0.26±0.06	0.51±0.04
α3β	22.2±8.0*	0.68±0.12

Figure S1. Picrotoxin sensitivity of recombinant GlyRs. Concentration-response curves of picrotoxin (PTX, 0.01 - 100 μM) were obtained using the EC_{50} of glycine for each GlyR subtype (α1: 45 μM, α3: 200 μM, α1β: 40 μM, α3β: 210 μM). The entire PTX concentration range was tested on each cell. Coexpression of the β subunit significantly reduced picrotoxin sensitivity of α1 and α3 GlyRs, confirming the presence of heteromeric channel complexes containing both α and β subunits. Data are mean ± SEM from 6 - 9 cells per group. *, $p < 0.05$, unpaired t-test.

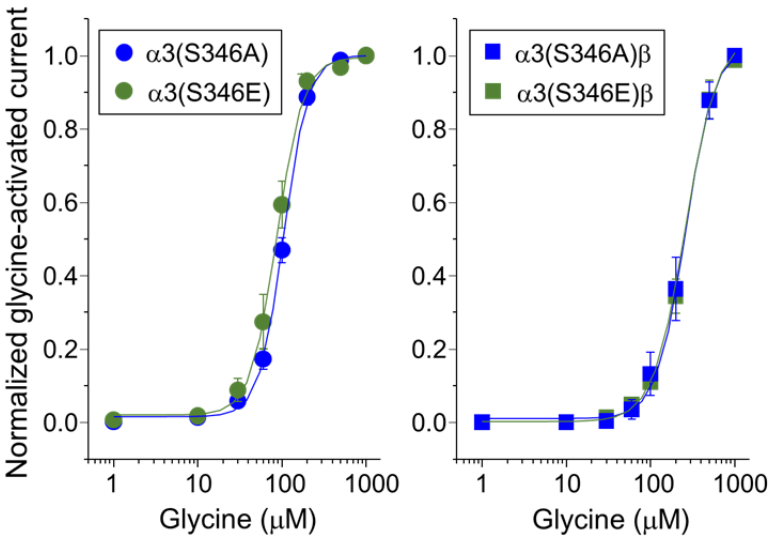


Figure S2. Glycine sensitivity of homomeric and α/β heteromeric α3(S346A) and α3(S346E) point mutated GlyRs expressed in HEK293T cells.

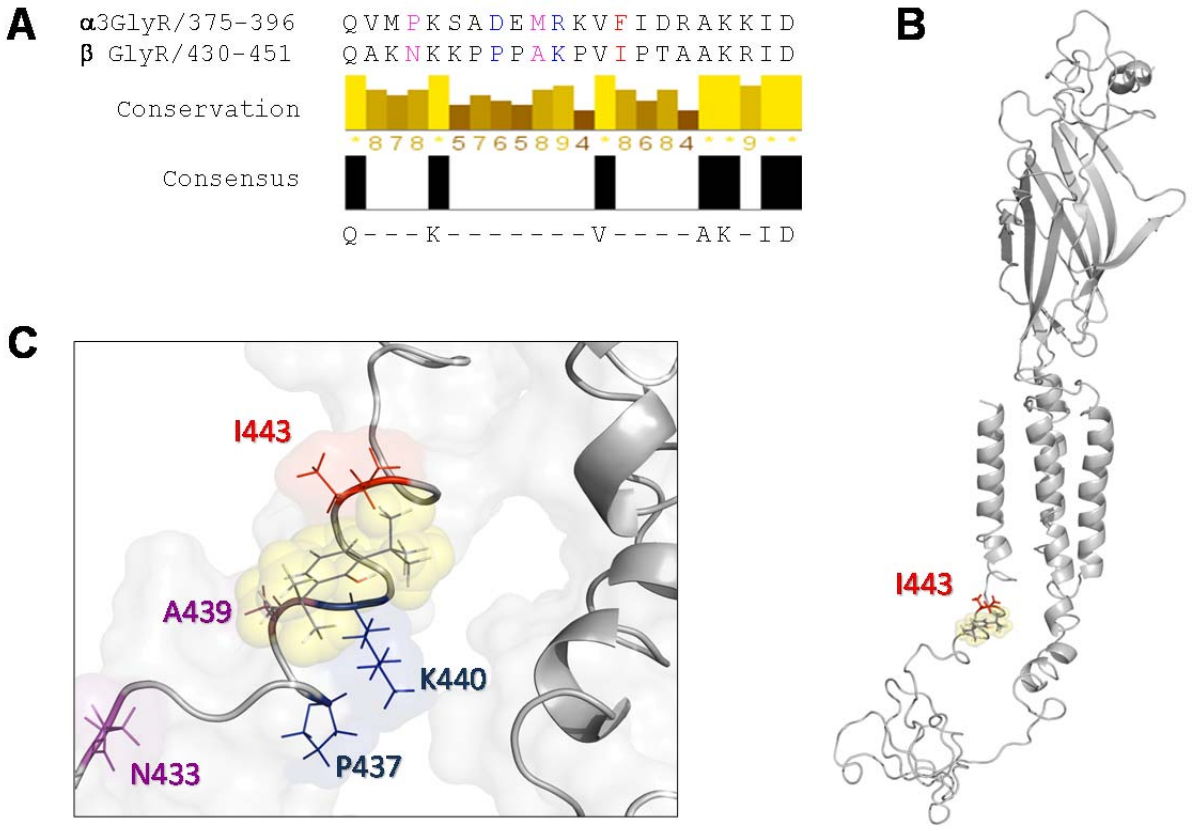


Figure S3. Structural model of GlyR β subunit. (A) Primary sequence alignment of the MA-stretch region of GlyRα3 and GlyR β subunits. The conservation scores are also shown (maximum 11, minimum 1). The identity percentage only reached 32%. **(B)** Homology model of β subunit monomer oriented in the plasma membrane. The location of the I443 residue (which is the counterpart of the F388 in α3GlyR) is highlighted in red. **(C)** Detailed view of the theoretical acceptor site of 2,6-DTBP in GlyR β

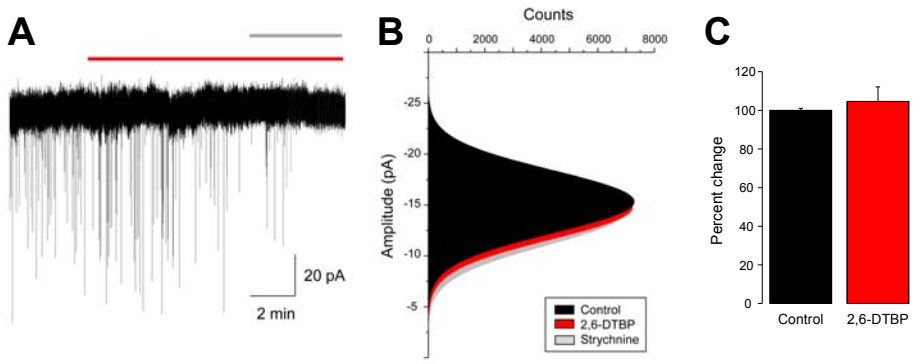


Figure S4. Effects of 2,6-DTBP on tonic glycinergic membrane currents lamina II neurons. Recordings were made in the presence of CNQX (5 µM), D-APV (50 µM) and bicuculline (10 µM). **(A)** Example trace during control conditions, after application of 2,6-DTBP (100 µM) and after additional application of strychnine (1 µM). **(B)** The all-point histograms indicate the holding current amplitudes under these three conditions. **(C)** 2,6-DTBP did not significantly affect the holding current (+4.6 ± 7.6% of control amplitudes, p = 0.56, paired t-test).

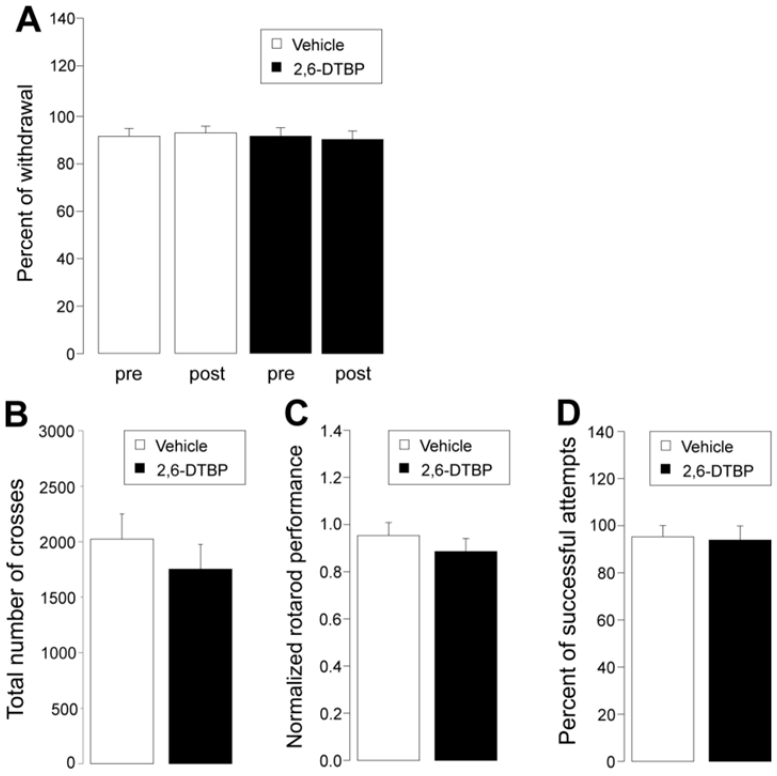


Figure S5. Effects of 2,6-DTBP on locomotor activity, motor coordination, muscle relaxation and acute pain. (A) 2,6-DTBP had no effect on acute nociceptive pain assessed in the pin-prick test in wild-type mice. (**B)** Locomotor activity (number of beam crosses in an open field arena, mean ± SEM) of wild-type mice after the administration of vehicle or 2,6-DTBP. n = 8 mice per group, p = 0.31, unpaired t-test. **(C)** Motor coordination (normalized time spent on an accelerating rotarod, mean ± SEM) of wild-type mice after the administration of vehicle or 2,6-DTBP (n = 7 – 9 mice per group, p = 0.39, unpaired t-test). **(D)** Muscle strength assessed in the horizontal wire test (percent number of successful attempts, mean ± SEM).

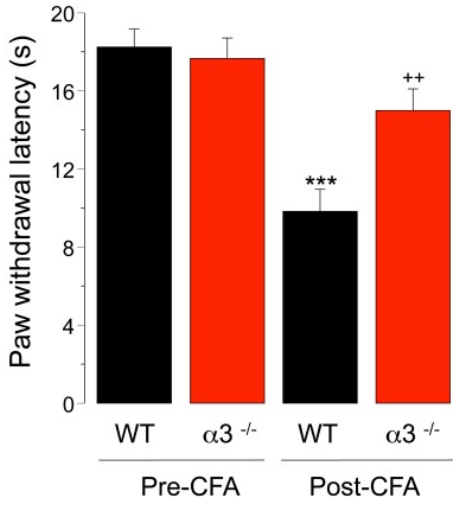


Figure S6. Effects of 2,6-DTBP on heat hyperalgesia in wild-type and in GlyRα3$^{-/-}$ mice after injection of CFA. Paw withdrawal latencies at baseline and 48 hrs after subcutaneous CFA injection. ANOVA followed by Bonferroni post-hoc test. F(3,61) = 10.38. ***, p < 0.001 WT pre-CFA versus WT post-CFA, $^{++,}$ p < 0.01, WT post-CFA versus GlyRα3$^{-/-}$ post-CFA.

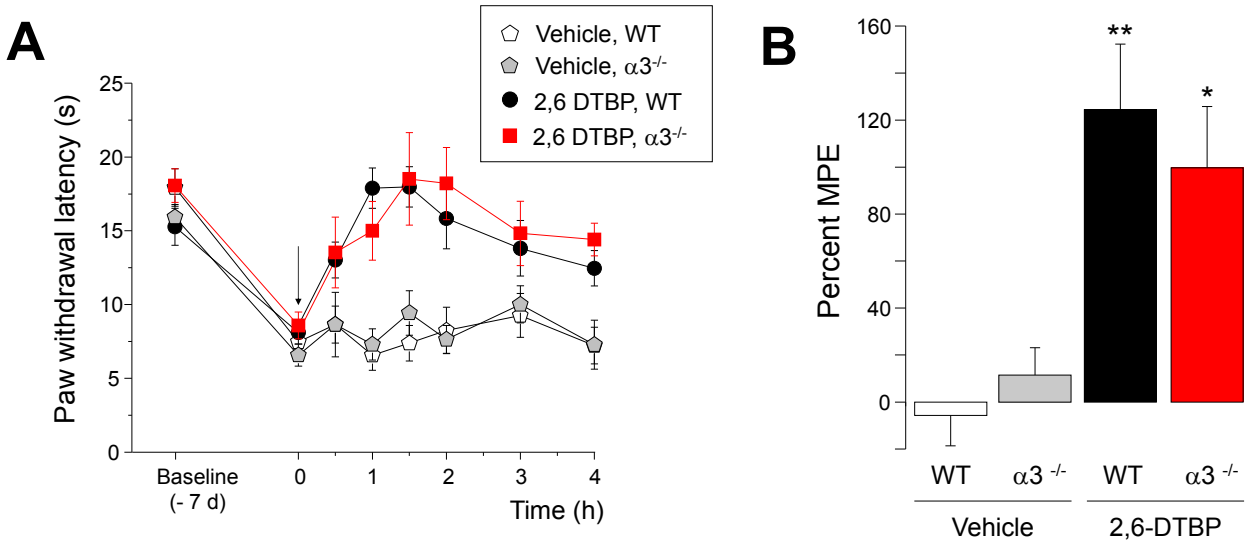


Figure S7. 2,6-DTBP effects on heat hyperalgesia in neuropathic (CCI) wild-type and GlyRα3$^{-/-}$ mice. (A) 2,6-DTBP or vehicle were applied (90 mg/kg, i.p.) on day 7 after CCI surgery. **(B)** ANOVA followed by Bonferroni post hoc test. F(3,25) = 9.42. **, p < 0.01, *, p < 0.05 before versus after 2,6-DTBP.

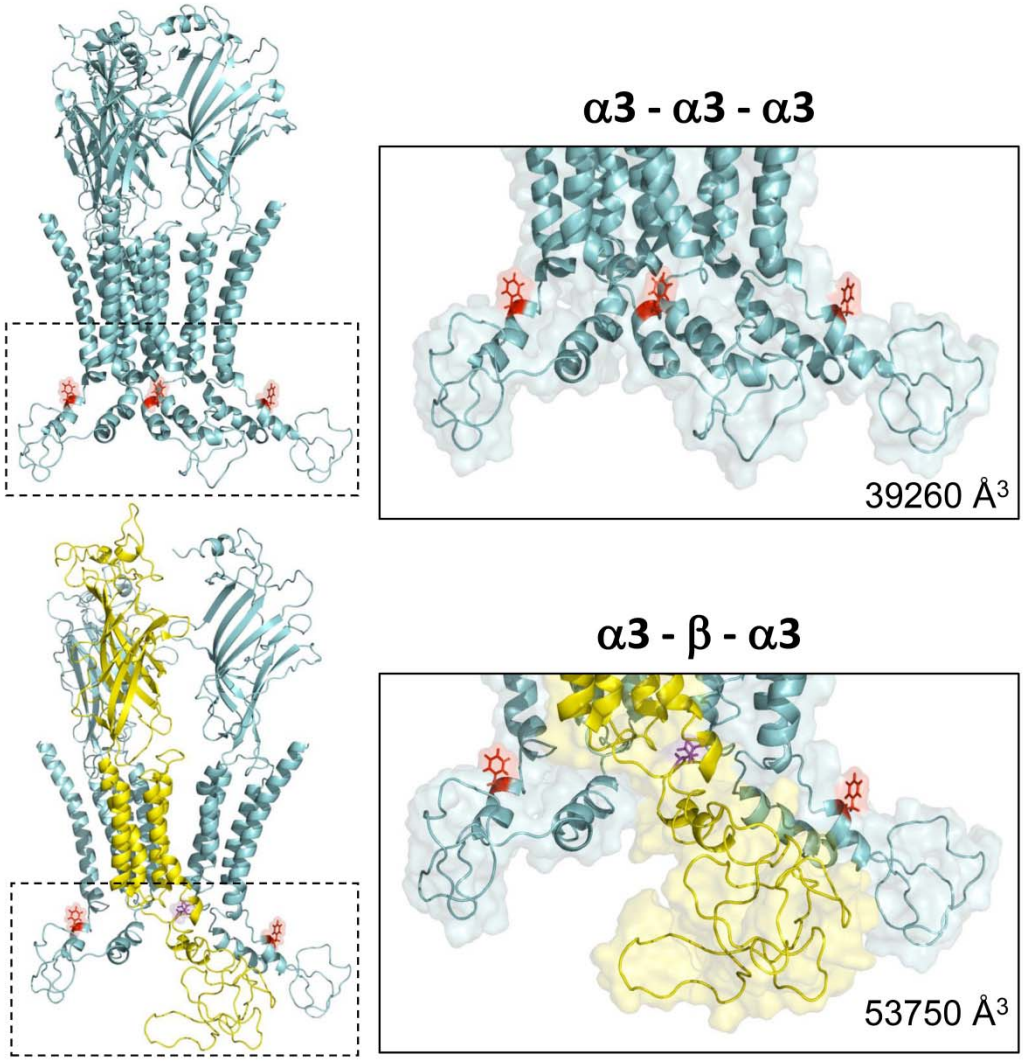


Figure S8. Structural models of the α3-α3-α3 and α3-β–α3 subunit interfaces. Graphic representation of a homotrimeric assembly composed of identical α3 subunits (upper panels, α3 subunits in cyan) and of a heterotrimeric assembly composed by a single β subunit (in yellow) flanked by two α3 subunits (lower panels). Right panels, magnifications of the regions indicated on the left encircling the putative acceptor sites for 2,6-DTBP in the GlyRα3 MA-stretch. The F388 residue in the GlyRα3 subunit and the I443 residue in the GlyR β subunit are highlighted in red and purple, respectively. Calculated volumes of the magnified regions are 39260 Å^3 and 53750 Å^3, for the α3-α3-α3 and the α3-β-α3 assemblies, respectively.

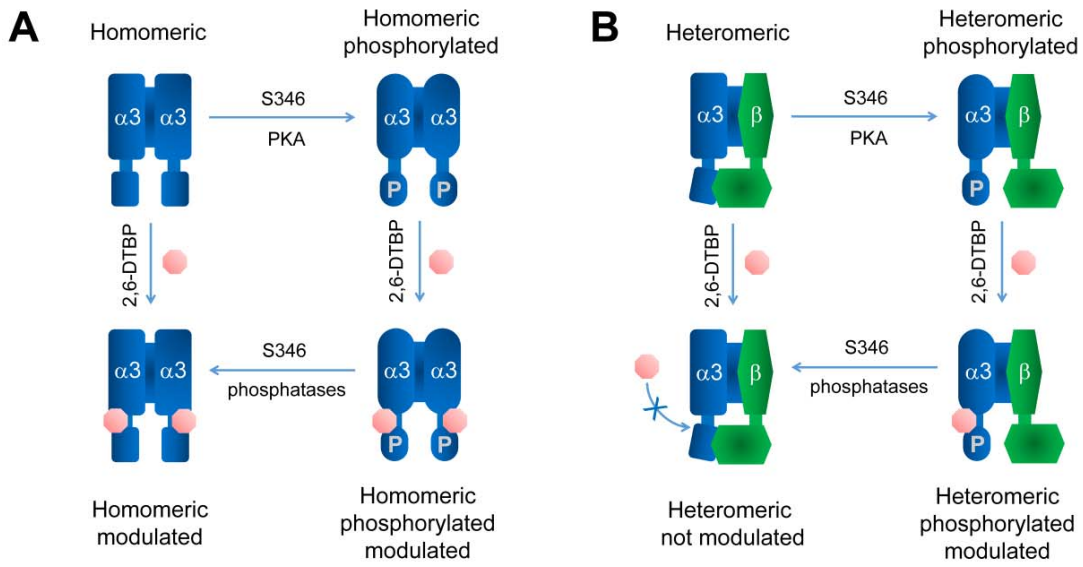


Figure S9. Molecular requirements for allosteric modulation by 2,6-DTBP of α3 and α3β GlyRs with phosphorylated or dephosphorylated GlyRα3 subunits. (A) Dephosphorylated homomeric GlyRα3 are positively modulated by 2,6-DTBP. PKA-dependent phosphorylation of S346 changes the GlyR conformation without preventing the modulation by 2,6-DTBP. **(B)** Heteromeric α3βGlyRs display a low sensitivity to modulation by 2,6-DTBP in their dephosphorylated state possibly due to the occlusion of the 2,6-DTBP interacting site by the β subunit.

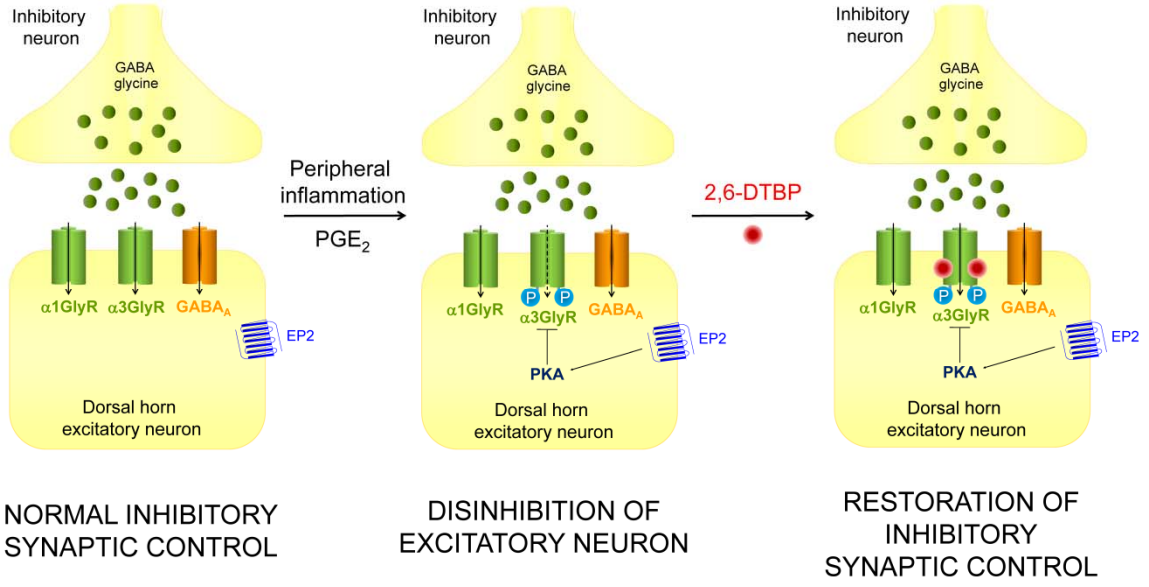


Figure S10. Proposed mechanism of the antihyperalgesic action for 2,6-DTBP. Presynaptic terminals of inhibitory interneurons release the neurotransmitters GABA and glycine into the synaptic cleft, allowing the phasic activation of postsynaptic $GABA_A$ and GlyRs. Under physiological conditions, the chloride influx through these ligand-gated ion channels provides efficient inhibitory control of excitatory neuron activity. Peripheral inflammation stimulates the production of spinal PGE_2, which subsequently activates EP2 receptors and stimulates PKA-dependent phosphorylation of synaptic GlyRs containing α3 subunits. Phosphorylation reduces the inhibitory postsynaptic currents through heteromeric α3βGlyRs and disinhibits dorsal horn neurons, which may contribute to the development and maintenance of hyperalgesic states. 2,6-DTBP selectively modulates synaptic α3βGlyRs in their phosphorylated states, allowing a restoration of the glycinergic control over the relay of nociceptive signals to higher CNS centers.

Table S1. Concentration-response curves of recombinant GlyRs expressed in HEK293T cells.

	EC_{50} (μM)	n_h	I_{max} (nA)	number of cells
α3	133±15	1.7±0.25	4.3±1.1	8
α1	54±3.0	2.1±0.21	4.2±0.8	9
α3β	117±5.0	3.0±0.32	4.0±1.0	9
α1β	98±4.0	1.9±0.12	4.6±1.0	6
α3 S346A	104±2.1	3.0±0.16	5.7±0.6	18
α3 S346E	101±2.6	2.7±0.17	3.9±0.7	16
α3β S346A	257±10.5	2.3±0.16	3.5±0.5	5
α3β S346E	259±11.0	2.5±0.21	4.3±0.6	5
α3 F388A	79±12.3	1.7±0.27	3.6±0.6	7
α3 S346E-F388A	125±8.1	1.54±0.13	2.9±0.8	3
α3β S346E-F388A	81±6.2	1.7±0.25	4.3±1.2	4

Concentration-response parameters were obtained from fits of normalized current amplitudes to the equation $I_{gly} = I_{max}[gly]^{nH}/([gly]^{nH} + [EC50]^{nH})$. The mean maximal current (I_{max}) indicates the average maximal current elicited by a saturating concentration of glycine (1 mM). Data were calculated from current responses activated by 1, 10, 30, 60, 100, 200, 500, and 1000 μM glycine recorded at a holding potential of -60 mV. All values are mean ± SEM.

Table S2. Single-channel kinetic parameters of wild-type and mutant α3GlyRs in the absence or the presence of 2,6-DTBP.

	GlyRα3							
	Glycine (100 μM)				Glycine (100 μM)/2,6-DTBP (10μM)			
	MOT (ms)	MST (ms)	nPo	Main γ (pS)	MOT (ms)	MST (ms)	nPo	Main γ (pS)
WT	21.6 ±6.0	310.0 ±60.5	0.20 ±0.02	91.0 ±1.20	55.6 ±8.19^{+}	139.2 ±21.5^{+}	0.58 ±0.10^{+}	90.1 ±0.92
F388A	19.1 ±3.2	201.6 ±51.9	0.18 ±0.05	88.2 ±1.82	18.1 ±4.89	213.6 ±27.8	0.19 ±0.04*	88.7 ±1.46

*, p <0.05, t-test, wild-type versus (F388A) point mutated α3GlyR.
$^{+}$, p < 0.05, paired t-test, control condition versus 2,6-DTBP
MOT, mean open time; MST, mean shut time; nPo, open probability; γ, conductance.

Table S3. Binding energy and docking scores of different propofol analogs on α3GlyRs.

	percentage potentiation on α3GlyR	docking score	ΔG binding (kcal/mol)	dock*ΔG index
2,4-di-tert-butylphenol	482±96	-2.79	-40.484	112.950
2,6-di-isopropyl-phenol (propofol)	442±99	-2.505	-38.708	96.962
2,6-di-tert-butylphenol	171±21	-2.133	-42.086	89.784
2,6-di-methoxi-phenol	3.0±20	-2.476	-32.177	79.669

Percent potentiation of wild-type α3GlyRs was calculated from experimental data using a modulator concentration of 100 μM. 2,6-di-methoxi-phenol was inactive on α3GlyRs and showed the lowest Dock*ΔG index. Dock*ΔG reflects the relationship between the docking attributes of each modulator with their binding energy at the respective site. Percent potentiation and the Dock*ΔG index show a linear correlation (r = 0.92, p = 0.08).

Chapter II: Pharmacological Modulation of Canonical and Non-Canonical Benzodiazepine-Binding Sites by Selected Benzodiazepine Site Ligands

In the following chapter, I describe the in vitro pharmacological profile of selected benzodiazepine-binding site ligands (BDZs) on $GABA_A$Rs. There are two major sections, one characterizing the pharmacological profile of BDZs with reduced sedative properties at canonical γ2-containing $GABA_A$Rs. In this section, I compare their differential sensitivity to all four BDZs sensitive subtypes (α1, α2, α3 and α5$GABA_A$Rs). In the second section, I characterize the sensitivity of selected BDZs at non-canonical benzodiazepine-binding sites, specifically those containing the γ1 subtype.

Part of this work has been published and is paraphrased from the original article:

*William T. Ralvenius, *Mario A. Acuña, Dietmar Benke, Alain Matthey, Youssef Daali, Uwe Rudolph, Jules Desmeules, Hanns Ulrich Zeilhofer, Marie Besson . The clobazam metabolite N-desmethyl clobazam is an α2 preferring benzodiazepine with an improved therapeutic window for antihyperalgesia. Neuropharmacology 2016.

*Co-first authors

ABSTRACT

Spinal disinhibition arises from different mechanisms including a decrease in GABAergic neurotransmission. Data from point mutated mice suggest that benzodiazepine-site agonists (BDZs) with preferential activity at α2-containing $GABA_ARs$ represent a useful tool for restoring spinal inhibition. Here I first analyzed the effect of clobazam (CBZ), its main metabolite N-desmethyl clomabzam (NDMC) and of diazepam (DZP) on recombinant $GABA_ARs$. DZP and CBZ potentiated α1$GABA_ARs$ and α2$GABA_ARs$ with similar efficacies, whereas NDMC showed higher efficacy at α2$GABA_ARs$ over α1$GABA_ARs$ across a wide concentration range. Then we studied the modulatory effects of a novel BDZ, HZ-166. This compound not only showed higher efficacy at α2$GABA_ARs$ over α1$GABA_ARs$, but also greater potency. Finally we assessed the potentiation profile of TP003 and TPA023B. TP003 displayed higher efficacy at α2 and α3$GABA_ARs$ over α1$GABA_ARs$. However, TPA023B showed the most profound difference with high potency and efficacy at α2 and α3$GABA_ARs$ and negligible effects at α1$GABA_ARs$. Although the γ2 subunit is required for full potentiation by BDZs, γ1-containing $GABA_ARs$ still possess sensitivity to certain BDZs. Moreover, previous data suggest that differences in the pharmacological effects of BDZs cannot only be explained based on their actions at the BDZ-sensitive α subunits. Therefore, I studied the modulatory activity of the BDZs at γ1$GABA_ARS$. Among all the BDZs tested HZ-166 showed the highest efficacy at α2β3γ1$GABA_ARs$. This effect was dependent on the BDZ-binding site at the α2 subunit. When tested at α1β2γ1$GABA_ARs$, HZ-166 showed negligible effects. Other results obtained by us and by other groups suggest that γ1 containing $GABA_ARs$ are expressed in the spinal dorsal horn. Together with these data, my results open the possibility that part of the analgesic action of HZ-166 and potentially of other BDZs originate from dorsal horn $GABA_ARs$ that contain γ1.

INTRODUCTION

Canonical BDZ-binding site: modulation of γ2-containing $GABA_A$Rs

In chronic pain states, the dorsal spinal cord undergoes plastic changes leading to the so-called disinhibition. Research of the last two decades has identified mechanisms underlying the reduction of inhibitory synaptic transmission in inflammatory or neuropathic pain states. i) In inflammatory pain, a prostaglandin E2-mediated phosphorylation of dorsal GlyRs renders these receptors less responsive to glycine (Ahmadi et al., 2002, Harvey et al., 2004). ii) Activated microglia down-regulate the expression of the potassium/chloride transporter KCC2 in superficial dorsal horn neurons after peripheral nerve injury (Coull et al., 2003, Coull et al., 2005, Keller et al., 2007). iii) Extensive nociceptor activation inhibits GABAergic interneurons (Pernia-Andrade et al., 2009). And iv) peripheral nerve damage leads to a down-regulation of the GABA synthesizing enzyme GAD65 in the spinal cord (Moore et al., 2002), and both inflammation and neuropathic nerve injury cause an epigenetic down-regulation of the same enzyme in the brainstem (Zhang et al., 2011).

$GABA_A$ receptors are heteropentameric ligand-gated ion channels. Although a very large number of genes encode for $GABA_A$R subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3), most of neuronal $GABA_A$Rs are composed of α, β, γ, or δ subunits in a 2α:2β:1γ or 2α:2β:1δ stoichiometry. The majority of $GABA_A$Rs in the CNS are sensitive to positive allosteric modulation by classical benzodiazepines-binding site ligands (BDZs), which bind to a site formed by one of four α subunits (α1, α2, α3 or α5) and the γ2 subunit (Wieland et al., 1992). Using point mutated mice carrying BDZ-insensitive α subunits, our group elucidated the contribution of the different α subunits to BDZ-mediated antihyperalgesia and to attribute this effect mainly to α2 and α3 $GABA_A$ receptors (Knabl et al., 2008). Moreover, taking advantage of the generation of triple $GABA_A$R point mutated mouse lines, we have found that the highest antihyperalgesic efficacy was attributable to α2-containing $GABA_A$Rs (α2$GABA_A$Rs) and adding activity at α3 or α5$GABA_A$Rs increases antihyperalgesic efficacy only moderately or not at all (Ralvenius et al., 2015). On the other hand, targeting α1$GABA_A$Rs leads to sedation and motor impairment. Therefore, subtype-selective BDZ agonists targeting only α2$GABA_A$Rs should have the best benefit-risk ratio.

In this chapter, I have analyzed the pharmacological profile of CBZ, NDMC, DZP, HZ-166, TP003 and TP023B. Previous studies have shown that the antihyperalgesic effects of clobazam (CBZ) - a licensed BDZ with reduced sedative properties - correlated better with blood levels of the main metabolite N-desmethyl clobazam (NDMC) than with blood levels of CBZ (Besson et al., 2013). The novel non-sedative 8-acetyleno-2'-pyridoimidazobenzodiazepine HZ-166 (Rivas et al., 2009) has been shown to exert

antihyperalgesia with no obvious sedative effects (Di Lio et al., 2011, Paul et al., 2014) and with better pharmacokinetic properties in mice than L-838,417 (Scott-Stevens et al., 2005). TP0A23B is a novel non-sedative anxiolytic imidazotriazine with negligible action at α1GABA$_A$Rs, modulating specifically those receptors containing the α2 and α3 subunit (Atack et al., 2011). TP003 is an anxiolytic compound, which has been shown to have specific modulatory activities only at α3GABA$_A$Rs and negligible efficacy at the α1, α2 and α5GABA$_A$Rs (Dias et al., 2005).

A thorough analysis of such compounds at all BDZ-sensitive GABA$_A$Rs will thus reveal a pharmacological profile aiming to find specific modulators for GABA$_A$Rs containing the α2 subunit, which mediates the antihyperalgesic action of BDZs.

Non-canonical BDZ-binding site: modulation of γ1-containing GABA$_A$Rs

The restoration of the spinal inhibitory pain control through the modulation of specific GABA$_A$R subunits is a new approach for pain therapy. So far, all the motivation has been placed on the study of selective modulators for different α subunits of the GABA$_A$Rs. Several lines of evidence from our lab suggest that differences in the pharmacological effects of BDZ site agonists cannot be explained solely on the basis of their differential actions at distinct α subtypes. Alternatively, some differences may originate from different subtypes of γ subunits included in the GABA$_A$R complex. HZ-166, acting on α2 or α3 containing GABA$_A$R, has analgesic effects, but is not muscle-relaxant (Di Lio et al., 2011, Paul et al., 2014). Flumazenil can antagonize the antihyperalgesic effect of spinally applied DZP only at high concentrations (Knabl et al., 2008). The specific ablation of γ2 subunit in primary nociceptors has no influence on the analgesic effects of spinally-applied DZP, although these effects are significantly reduced when α2 is ablated (Witschi et al., 2011). In mice carrying the H → R point mutation in the α2 subunit, which renders α2GABA$_A$Rs insensitive to BDZs, the antihyperalgesic effects of spinally-applied DZP were reduced by about 80% (Knabl et al., 2008), but spinal flumazenil binding sites are reduced only by < 10% (Ralvenius et al., 2015). DMCM, an antagonist at γ2-GABA$_A$Rs but agonist at γ1-GABA$_A$Rs, displays antihyperalgesic effects in neuropathic & inflammatory pain models (Munro et al., 2011).

Remarkably, among the three known γ subunits, γ2 is the most expressed in the brain (Mohler et al., 2002, Fritschy and Brunig, 2003). The possibility of targeting 'non-γ2'-GABA$_A$Rs might therefore lead to a selectivity of GABA$_A$R modulation and thus establish a new possibility for spinal GABA$_A$R-mediated analgesia. Although classical BDZs require the γ2 subunit for full potentiation, γ1 and γ3-containing GABA$_A$Rs are still sensitive to certain BDZ site ligands (McKernan et al., 1995, Esmaeili et al., 2009). Therefore, we aimed to

pharmacologically characterize the sensitivity of γ1-containing $GABA_ARs$ to different BDZ-site ligands, namely DZP, HZ-166, CGS-20625, TPA023B and NDMC.

METHODS

Electrophysiology. The effects of DZP, CBZ, NDMC, HZ-166, TPA023B and TP003 on currents through recombinant $GABA_AR$s were studied in HEK293 cells transiently transfected with rat $GABA_AR$ subunits using lipofectamine LTX 46 (Invitrogen). To ensure expression of the γ2 or γ1 subunit in all recorded cells, we transfected cells with a plasmid expressing the γ1/2 subunit plus eGFP from an IRES, and selected only eGFP-positive cells for recordings (see also (Ralvenius et al., 2015)). The transfection mixture contained (in µg): 1 αx, 1 βy, 3 γ1-2/eGFP. Whole-cell patch-clamp recordings were made 18 – 36 hrs after transfection at room temperature (20 – 24°C) and at a holding potential of -60 mV. Recording electrodes were filled with solution containing (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.40), 4 MgCl2, 0.5 GTP and 2 ATP. The external solution contained (in mM): 150 NaCl, 10 KCl, 2.0 CaCl2, 1.0 MgCl2, 10 HEPES (pH 7.40), and 10 glucose. GABA was applied to the recorded cell using a manually controlled pulse (6 - 10 s) of a low sub-saturating GABA concentration (EC_{5-10}). EC_{5-10} values of GABA applied for the four canonical benzodiazepine-sensitive $GABA_AR$ combinations (α1β2γ2, α2β3γ2, α3β3γ2 and α5β2γ2) and non-canonical benzodiazepine γ1-containing $GABA_AR$s (α1β2γ1, α2β3γ1, α3β2γ1). EC_{50} values and Hill coefficients (nH) were obtained from fits of normalized concentration-response curves to the equation $I_{GABA} = I_{max}\ [GABA]^{nH} / ([GABA]^{nH} + [EC50]^{nH})$. I_{max} was determined as the average maximal current elicited by saturating concentration of GABA (30 µM – 3 mM, depending on the subunit composition). DZP, CBZ, NDMC, HZ-166, TP003 and TPA023B, CGS 20625 were dissolved in DMSO (final concentration < 0.1%) and subsequently diluted on the day of the experiment in external solution and were co-applied with GABA without preincubation. Concentration-response curves were fitted using the equation for sigmoidal concentration-response curve with variable slope: $E(C) = (E_{max} \cdot [C]^{nH}) / ([C]^{nH} + [EC50]^{nH})$.

RESULTS

Characterization of canonical BDZ-sensitive $GABA_AR$s

Modulatory effects of DZP, CBZ and NDMC at all BDZ-sensitive $GABA_AR$s[1]

In this section, I compared the pharmacological profile of the CBZ metabolite NDMC with CBZ and DZP. More specifically I analyzed the positive allosteric modulation by these compounds on recombinant $GABA_AR$s expressed in HEK293 cells (Figure 1). In electrophysiological experiments, all three compounds potentiated currents through α1β2γ2, α2β3γ2, α3β3γ2 and α5β2γ2$GABA_AR$s (short α1-, α2-, α3- and α5$GABA_AR$s) but did not directly activate $GABA_AR$ currents at the concentrations tested (up to 3 μM for DZP, and 20 μM for CBZ and NDMC). Differences were observed with respect to the potency and efficacy of the three compounds at the four $GABA_AR$ subtypes. DZP potentiated currents through all four subtypes with EC_{50} values between 0.029 and 0.071 μM (Figure 1B). CBZ and NDMC were less potent with EC50 values between 0.40 and 1.1 μM and between 0.49 and 0.81 μM, respectively (Figure 1C,D). Pronounced differences were found when comparing the efficacy of potentiation by the three compounds at the different $GABA_AR$ subtypes. Potentiation by DZP was strongest for α3$GABA_AR$s, while potentiation of the other three $GABA_AR$ subtypes ranged between 100% and 141%. At concentrations < EC_{50}, which are probably more relevant to therapeutic effects of DZP, α1$GABA_AR$s were potentiated more strongly than α2-, α3- and α5$GABA_AR$s. CBZ and DZP had very similar efficacies at the four $GABA_AR$ subtypes, but CBZ differentiated less between subtypes at sub-saturating concentrations. NDMC potentiated α2 and α3GABAARs to a considerably higher degree (253 and 245%) than α1 and α5 (143 and 148%) (Figure 1D). This potentiation was negligible at $GABA_AR$s carrying the BDZ-insensitive (α2H-R$GABA_AR$S) subunit (Figure 2). Taken together, DZP preferred α1$GABA_AR$s at low concentrations and α3$GABA_AR$s at high concentrations. CBZ was rather non-specific at low concentrations and preferred α3$GABA_AR$s at high concentrations, while NDMC showed the strongest potentiation at α2$GABA_AR$s over the concentration range tested.

[1] This section, including Figure 1, was reproduced from its original version published in *Neuropharmacology* by Ralvenius WT & Acuña MA et al., 2016.

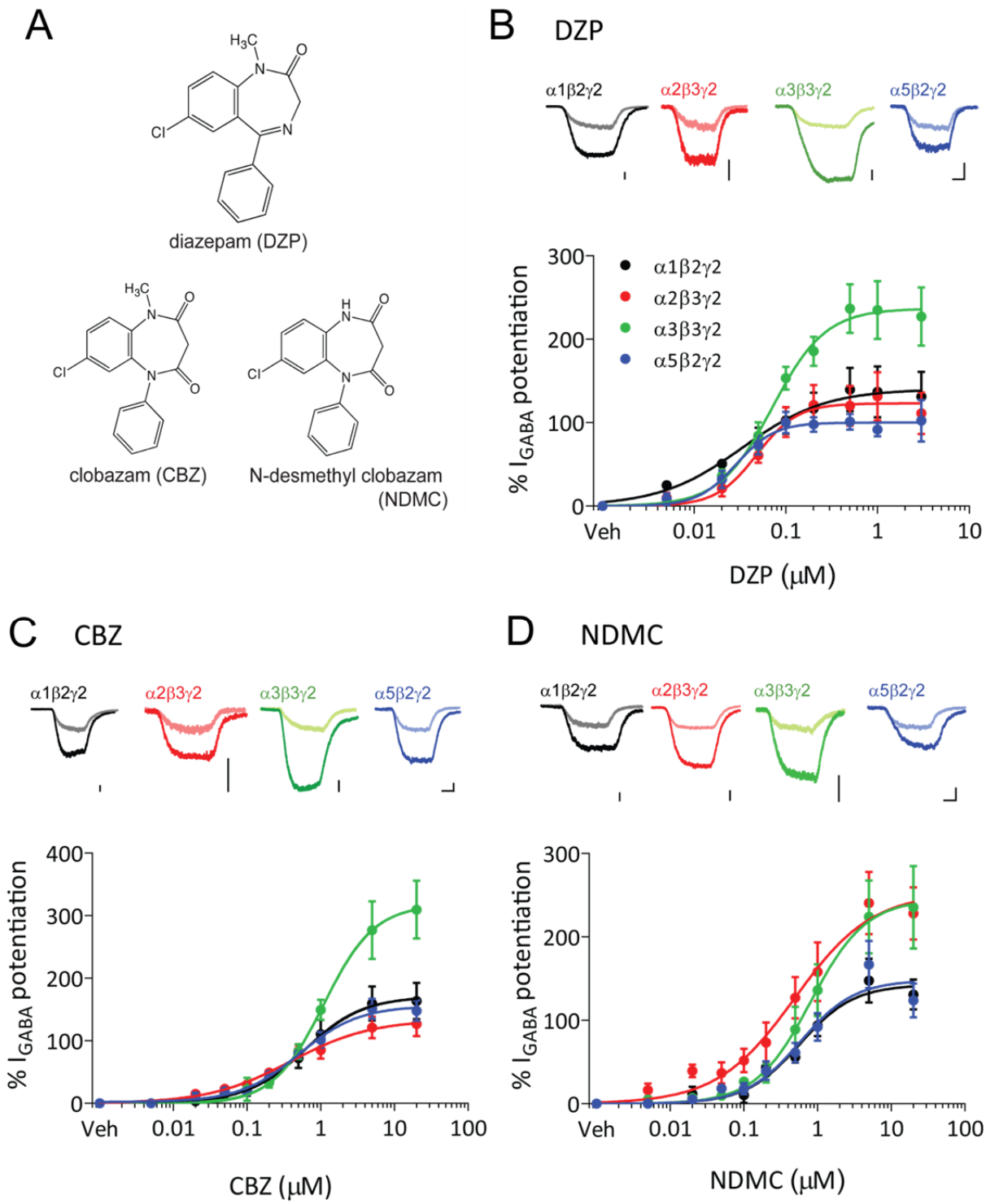


Figure 1. Potentiating effects of DZP, CBZ and NDMC on the four BDZ-sensitive $GABA_AR$ subtypes. **(A)** Chemical structure of DZP, CBZ, and NDMC. **(B-D)** GABA-evoked membrane currents were measured in HEK293 cells transiently transfected with recombinant α1β2γ2 (grey and black), α2β3γ2 (light and dark red), α3β3γ2 (light and dark green), and α5β2γ2 (light and dark blue) $GABA_ARs$. Top panels, traces show current responses evoked by GABA before and during application of a saturating concentration of DZP, CBZ, or NDMC (1 μM in case of DZP, and 20 μM in case of CBZ and NDMC). Light and dark traces before and during application of the BDZ, respectively. GABA was applied at EC_{10} in all experiments (1 μM, 5 μM, 8 μM, and 1 μM, for α1β2γ2, α2β3γ2, α3β3γ2, and α5β2γ2 GABAARs, respectively) for 6 -10 s. Scale bars, 2 s and 200 pA. Bottom panels, concentration response curves of DZP, CBZ and NDMC obtained for the four $GABA_AR$ subtypes at EC10 of GABA.

Data are mean ± SEM. Curves represent fits to Hill's equation with a baseline fixed to 0. n, numbers of cells = 5 – 7 for all data points.

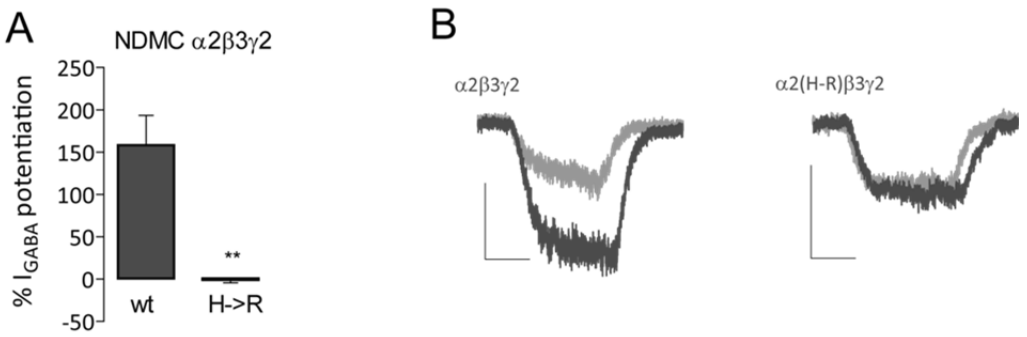


Figure 2. Lack of modulation by NDMC (1 μM) of α2(H-R)β3γ2 point mutated $GABA_AR$s. **(A)** Loss of sensitivity in the point mutated α2(H-R)β3γ2 $GABA_AR$. n = 5 per condition. Unpaired t test **, P < 0.01. Data are mean ± SEM. **(B)** Representative GABA (EC_{10})-evoked traces and NDMC (1 μM) in WT α2β3γ2$GABA_AR$s and BDZ-insensitive α2(H-R)β2γ2$GABA_AR$s. Grey and black traces indicate before and during application of NDMC, respectively. Scale bars, 2 s and 200pA.

$GABA_AR$ modulation by HZ-166

Using the same methodology as in the previous section, I studied the effect of HZ-166 as positive allosteric modulator of $GABA_AR$s expressed in HEK293 cells (Figure 3). HZ-166 potentiated all four combinations of $GABA_AR$s, although differences were observed in terms of potency and efficacy at the four $GABA_AR$ subtypes. HZ-166 showed the highest potency at α2$GABA_AR$s (EC_{50} (mean ± SD) value of 0.167 ± 0.0107 μM), which is about 8-times higher than at α1$GABA_AR$s (EC_{50} (mean ± SD) value of 1.3 ± 0.7 μM). In the case of α3 and α5$GABA_AR$s, HZ-166 showed similar potency at those combinations (EC_{50} (mean ± SD) values for α3 of 0.50 ± 0.112 μM and for α5 of 0.47 ± 0.089 μM). At concentrations < EC_{50}, which are probably more relevant to therapeutic effects, α2- and α3- $GABA_AR$s were potentiated more strongly than α1 and α5$GABA_AR$s. Similarly to NDMC, HZ-166 potentiated α2 and α3$GABA_AR$s to a higher degree (155.1 ± 15 and 184.8 ± 12%, mean ± SEM) than α1 and α5$GABA_AR$S (116.6 ± 10% and 112 ± 21%, mean ± SEM) at saturating concentrations. These data indicate that HZ-166 preferred α2 and α3$GABA_AR$s with high potency and efficacy, making it an interesting candidate for future behavioral studies on pain.

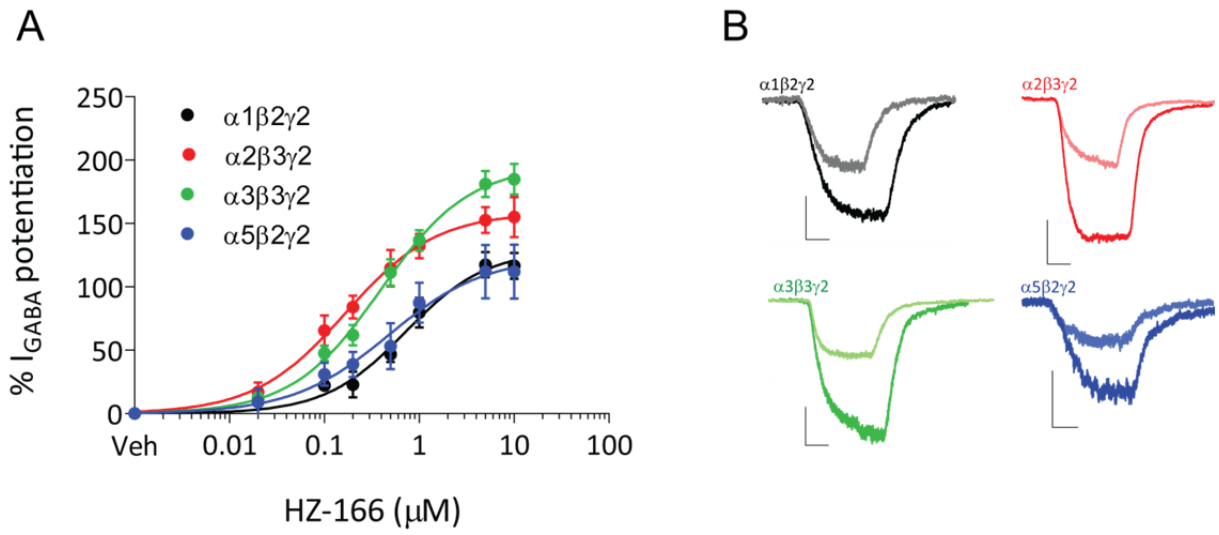


Figure 3. Modulation of $GABA_A$Rs by HZ-166. (A) Concentration-response curves for HZ-166 obtained for α1β2γ2 (black), α2β3γ2 (red), α3β3γ2 (green) and α5β2γ2 (blue). Data are mean ± SEM. Curves represent fits to Hill's equation with a baseline fixed to 0. n, numbers of cells, = 5 – 6 for all data points. **(B)** GABA-evoked membrane currents were measured in HEK293 cells transiently transfected with recombinant α1β2γ2 (grey and black), α2β3γ2 (light and dark red), α3β3γ2 (light and dark green), and α5β2γ2 (light and dark blue) $GABA_A$Rs. Scale bars, 2 s and 200pA. GABA at EC_5 was applied in all experiments.

$GABA_A$R modulation by TPA023B and TP003

I next analyzed the effects of two novel BDZ site ligands, TPA023B (Atack, Hallett et al. 2011) and TP0003 (Dias, Sheppard et al. 2005) in recombinant $GABA_A$Rs. Although these compounds have been characterized before, I considered that it is important to replicate the earlier studies in order to obtain own data on the actions of these drugs. Moreover, a recent study has shown conflicting results regarding the specificity of TP003 acting at α3$GABA_A$Rs. It has been shown that TP003 unexpectedly potentiated $GABA_A$Rs at all four BDZ-sensitive combinations (de Lucas et al., 2015) contradicting what was found previously (Dias et al., 2005). Therefore, I decided to perform in-house characterization of the modulatory activity of TPA023B and TP003. TP003 shows higher potency at α3$GABA_A$Rs compared to the α1, α2, and α5$GABA_A$Rs (EC_{50} (mean ± SD) values for α1, α2, α3 and α5$GABA_A$Rs: 20 ± 3.2 nM, 10 ± 3 nM, 3 ± 1 nM and 5.6 ± 1 nM, respectively) (Figure 4A). This compound also shows higher efficacy at α3$GABA_A$Rs over the range of concentration tested, but similar efficacies at α2 and α5$GABA_A$Rs with lower efficacy at α1$GABA_A$RS. Conversely, TPA023B showed efficacy only at α2 and α3$GABA_A$Rs, with small potentiation at α5 (only at saturating concentrations) and no activity at α1 (Figure 4B). Although this compound showed lower potency at α2 and α3$GABA_A$Rs than TP003, it remarkably exhibited a preferential action on α2/3 with negligible efficacy at α1$GABA_A$Rs. Modulatory activity of TPA023B, in terms of efficacy and potency, at α2 was comparable to α3$GABA_A$Rs (EC_{50} (mean ± SD). Values for

α2 and α3GABA$_A$Rs are 51 ± 18 nM and 35 ± 10 nM, respectively; and maximal efficacy values are 107.3 ± 18% and 109.4 ± 24%, respectively).

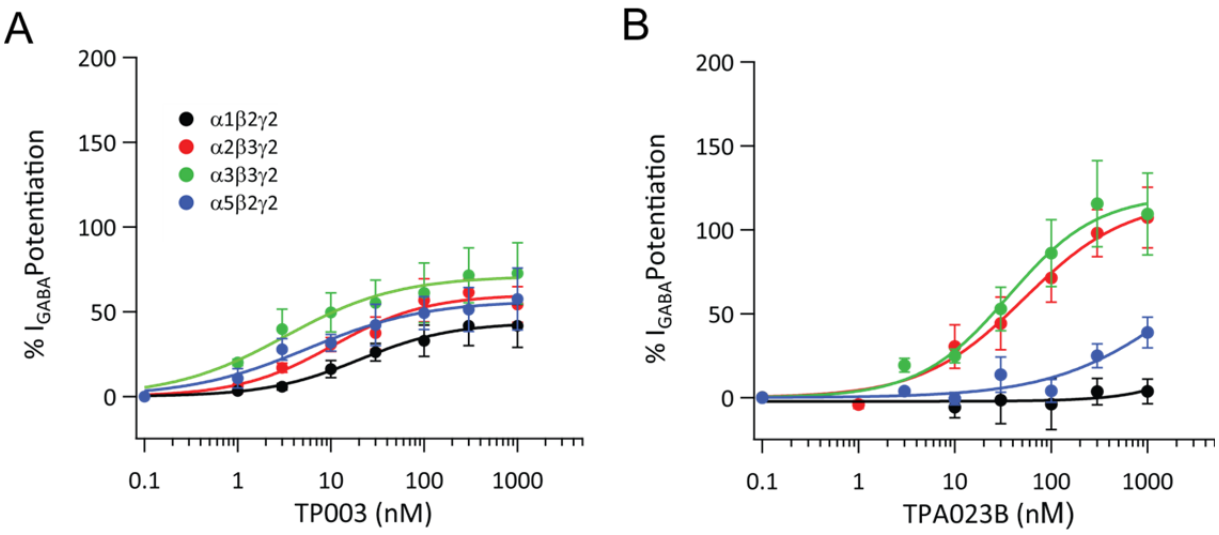


Figure 4. Potentiating effects of TP003 and TPA023B on the four BDZ-sensitive GABA$_A$Rs. Concentration-response curves obtained for α1β2γ2 (black), α2β3γ2 (red), α3β3γ2 (green) and α5β2γ2 (blue) for TP003 **(A)** and TPA023B **(B)**. Data are mean ± SEM. Curves represent fits to Hill's equation with a baseline fixed to 0. n, numbers of cells, = 5 – 6 for all data points.

Characterization of non-canonical BDZ-sensitive $GABA_AR$s

To characterize the sensitivity to selected BDZs of γ1 versus γ2-containing $GABA_AR$s, I performed whole-cell patch clamp experiments in HEK239 cells expressing either αx/βy/γ2 $GABA_AR$s or αx/βy/γ1 $GABA_AR$s. The first step was to test whether γ1 and γ2-containing $GABA_AR$s were functionally assembled at the cell membrane. We took advantage of the differential sensitivity to Zn^{2+} of receptors containing or not γ subunits (Draguhn et al., 1990, Smart et al., 1991). α2/β3 subunit combinations showed high sensitivity, whereas the inclusion of any γ subunit to the receptor complex rendered the receptor less sensitive to the inhibitory effects of Zn^{2+} (Figure 5) (IC_{50} values for α2β3$GABA_AR$s and α2β3γ1/2$GABA_AR$s are (mean ± SD) 0.39 ± 0.16 μM, 32 ± 1.7 μM and 131 ± 136 μM, respectively). These data thus verify the incorporation of the γ1 subunit into the $GABA_AR$ complex.

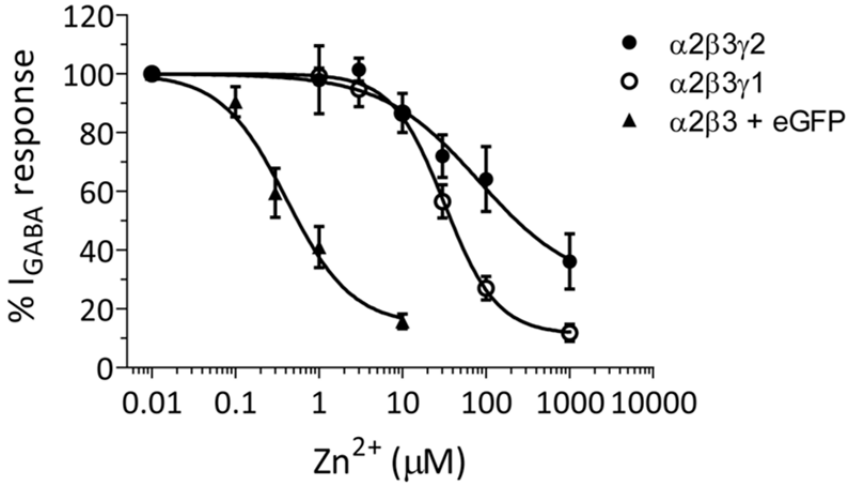


Figure 5. Zn^{2+} potency is affected by the inclusion of γ1 and γ2 subunits in the $GABA_AR$ complex. Concentration inhibition curves determined for α2β3, α2β3γ1 and α2β3γ2$GABA_AR$s using an EC_{50} of GABA. Data was fitted to Hill's equation n = 3 – 6 cells for all data points.

I further characterized different BDZs, namely diazepam, HZ-166, CGS20625, TPA023B and NDMC at α2β3γ1$GABA_AR$s and compared the sensitivity to α2β3γ2$GABA_AR$S. We selected only the α2-containing $GABA_AR$s, because these receptors are the most relevant for spinal $GABA_AR$s-mediated antihyperalgesia (Knabl et al., 2008, Ralvenius et al., 2015). All of the drugs tested potentiated $GABA_AR$s in the configuration α2β3γ1 (Figure 6A-C). DZP showed lower potency (EC_{50} (mean ± SD) values are 30.8 ± 6.98 μM and 0.12 ± 0.01 μM, for α2β3γ1 and α2β3γ2$GABA_AR$S, respectively) but similar efficacy as α2β3γ2 at each corresponding maximal concentration tested (Figure 6A) (E_{max} (mean ± SEM) values are 101 ± 12% and 94 ± 16.5% for α2β3γ1 and α2β3γ2$GABA_AR$s, respectively). Unexpectedly, at saturating concentrations, the efficacy by HZ-166 was stronger at γ1-containing $GABA_AR$s than at γ2$GABA_AR$s (E_{max} (mean ± SEM) values are 374.7 ± 108% and 162.7 ± 19.5% for α2β3γ1 and α2β3γ2$GABA_AR$s, respectively), whereas it showed lower potency at γ1$GABA_AR$s (EC_{50}

(mean ± SD) values are 8.8 ± 2.3 μM and 0.15 ± 0.01 μM, for α2β3γ1 and α2β3γ2$GABA_A$Rs, respectively). Moreover, this modulation was dependent on the presence of the γ subunit, since HZ-166 had negligible activity at α2β3$GABA_A$R (0.5 ± 7% vs 374.7 ± 108% in α2β3 versus α2β3γ1$GABA_A$Rs, respectively; unpaired t-test $p<0.05$, n = 3 - 5). CGS 20625, a compound that has positive allosteric modulation activity at α1β2γ1 (Khom et al., 2006), showed increased efficacy at α2β3γ1 at low concentrations over α2β3γ2$GABA_A$Rs. TPA023B differentiated strongly between subtypes, acting with lower potency (EC_{50} (mean ± SD) values are 0.74 ± 0.01 μM and 0.055 ± 0.02 μM, for α2β3γ1 and α2β3γ2$GABA_A$RS, respectively) and decreased efficacy (E_{max} (mean ± SEM) values are 54.1 ± 6.7% and 107 ± 18% for α2β3γ1 and α2β3γ2$GABA_A$Rs, respectively). Pronounced differences were also found when comparing the efficacy and potency by NDMC at γ1 and γ2$GABA_A$Rs. Potentiation by NDMC was stronger at α2β3γ2 than α2β3γ1$GABA_A$RS, showing higher potency (EC_{50} (mean ± SD) values are 2.5 ± 3.4μM and 0.58 ± 0.27 μM, for α2β3γ1 and α2β3γ2$GABA_A$RS, respectively) and efficacy (E_{max} (mean ± SEM) values are 79.6 ± 6.7% and 198 ± 48.7% for α2β3γ1 and α2β3γ2$GABA_A$Rs, respectively).

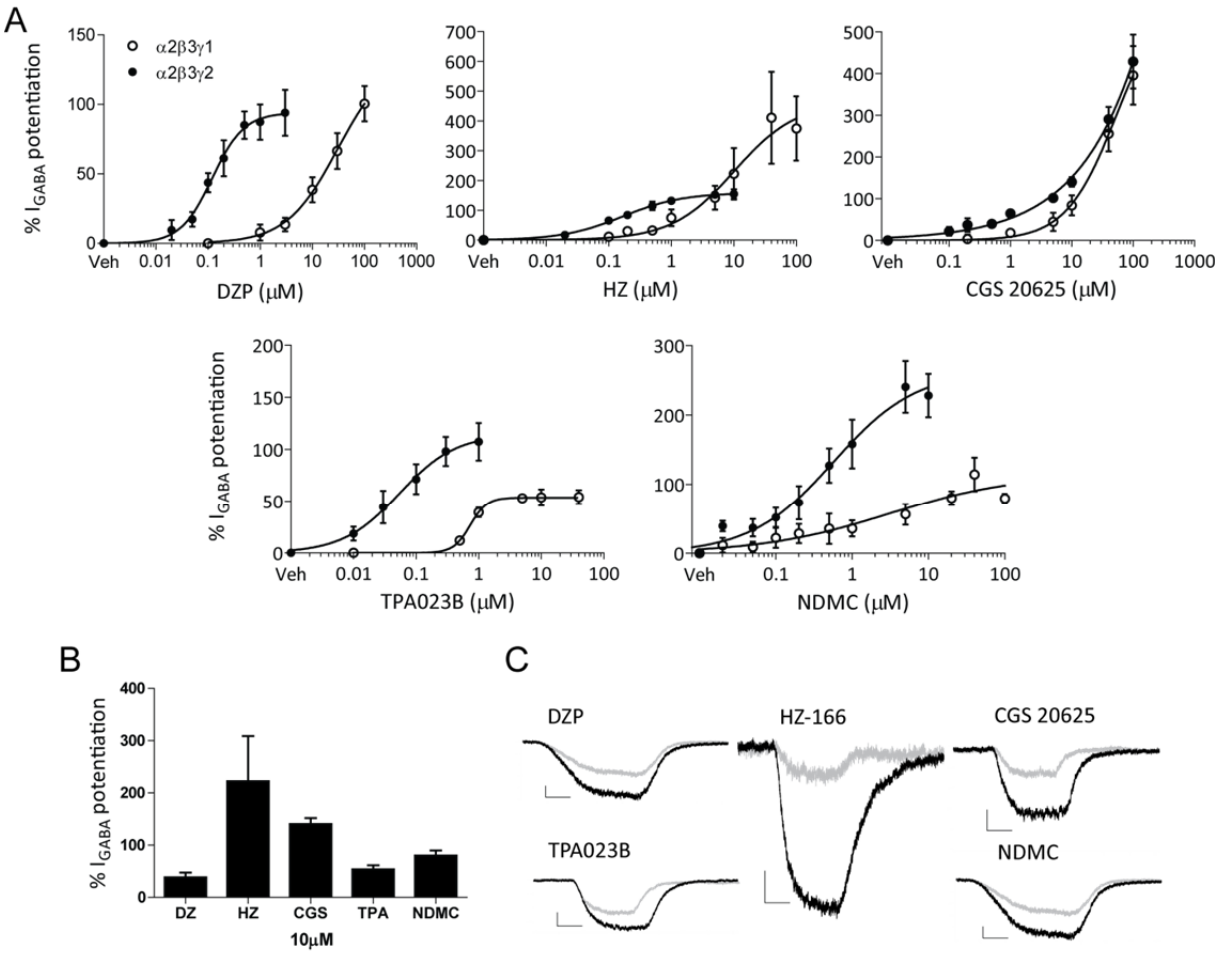


Figure 6. Potentiating effects of GABA-induced membrane currents by DZP, HZ-166, CGS 20625, TPA023B and NDMC on α2β3γ1GABA$_A$Rs. (A) Concentration response curves of DZP, HZ-166, CGS 20625, TPA023B and NDMC compared to the modulation of α2β3γ2GABA$_A$RS. **(B)** Efficacy of potentiation of GABA-induced current (EC_5) by 10μM of each compound at α2β3γ1GABA$_A$Rs. Data are mean ± SEM. n = 3 - 8 cells **(C)** GABA-evoked membrane currents of recombinant α2β3γ1GABA$_A$Rs. Grey and black traces indicate before and during application of the BDZ, respectively. Scale bars 200 pA, 2s.

Given the high efficacy elicited by HZ-166 at α2β3γ1GABA$_A$Rs (Figure 6B-C), and the necessity of the presence of a γ subunit, we wondered whether this potentiation is dependent on the BDZ-binding site. We therefore investigated the potentiation of HZ-166 at α2β3γ1 and the BDZ-insensitive mutant α2(H-R)β3γ1 GABA$_A$Rs. HZ-166 showed a strong decrease of efficacy in the point mutated receptors (Figure 7A-B). Moreover, when we expressed α1β2γ1GABA$_A$Rs, the modulatory effects of HZ-166 were comparable to those found in α2 point mutated receptors (Figure 7A-B). These results strongly suggest that the efficacy of potentiation by HZ-166 at γ1GABA$_A$Rs depends on the BDZ-binding site at the α subunit and that, in the presence of the α1 subunit, the potentiation is virtually lost. Additionally, the inclusion of an α3 subunit in the receptor complex does not affect the modulatory effects of HZ-166 at γ1-containing GABA$_A$Rs (Figure 7).

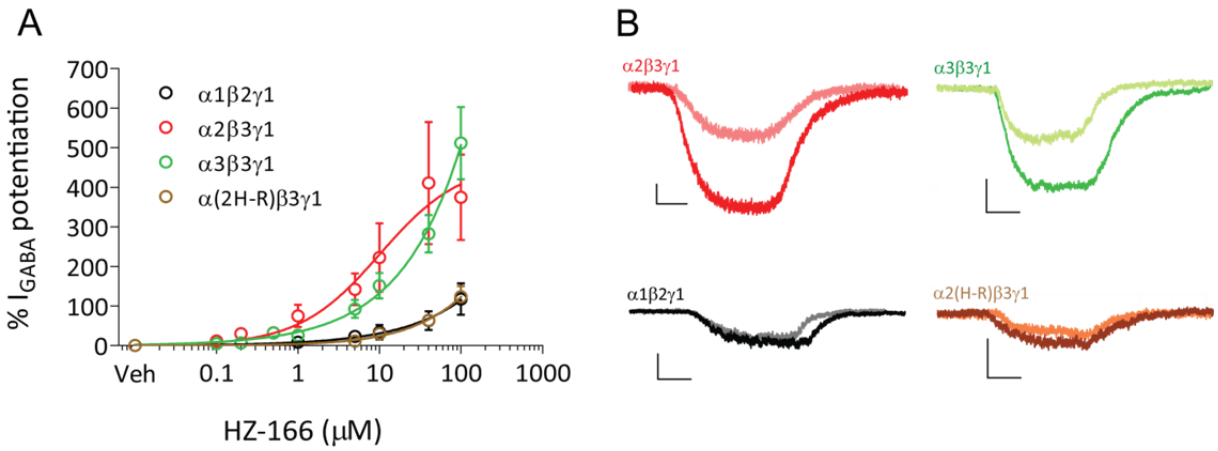


Figure 7. BDZ-binding site dependency of modulation by HZ-166. **(A)** Concentration response curve obtained for α2β3γ1, α3β3γ1, α1β3γ1 and α2(H-R)β3γ1 $GABA_A$Rs. Data are mean ± SEM. Curves represent fits to Hill's equation with a baseline fixed to 0. **(B)** GABA-evoked membrane currents of recombinant α1β2γ1 (grey and black), α2β3γ1 (light and dark red), α3β3γ1 (light and dark green) and α2(H-R)β3γ1 (light brown and dark brown) $GABA_A$Rs. Light and dark traces represent before and during application of HZ-166 (10μM), respectively. Scale bars, 2 s and 200 pA.

DISCUSSION

Targeting $GABA_A$ receptors is a new approach to restore spinal inhibition, which is a hallmark of persistent pain states. Moreover, there is increasing evidence from preclinical studies to suggest that non-sedative benzodiazepines with improved subunit selectivity exert antihyperalgesic actions. Indeed, strong antihyperalgesia with low sedative effects can be achieved by targeting α2-containing $GABA_ARs$ and avoiding activity at α1$GABA_ARs$. My work has therefore focused on finding positive allosteric modulators for $GABA_ARs$ with a high α2/α1 selectivity ratio. The classical BDZ-binding site of $GABA_ARs$ lies at the interface between an α1, α2, α3, or α5 and the γ2 subunit. We first studied the potentiating effects of NDMC, which is the main metabolite of the licensed antiepileptic drug CBZ, and compared it to the effects of DZP. At saturating concentrations, DZP and CBZ potentiated α2 and α1$GABA_ARs$ subtypes with similar efficacy, whereas NDMC clearly favored α2 over α1$GABA_ARs$. At low concentrations (< EC_{50}), NDMC preferentially modulated with higher efficacy at α2$GABA_ARs$ across the entire concentration range tested, whereas CBZ had similar effects on α2 and α1$GABA_ARs$ and DZP favored α1$GABA_ARs$. Therefore, we show here that NDMC possesses an improved α2/α1 ratio. Our group has recently published these results together with an *in vivo* analysis of the pharmacological profile of NDMC (Ralvenius et al., 2016). We demonstrated that NDMC has a more favorable pharmacological profile than classical BDZ, suggesting that NDMC might represent a useful compound for initiating proof-of-concept trials in healthy volunteers or chronic pain patients.

A higher α2/α1 ratio was also evident for HZ-166. This compound showed higher potency and efficacy at α2 compared to α1 for all concentrations tested. HZ-166 has been shown to have antihyperalgesic actions in several chronic pain models (Di Lio et al., 2011, Paul et al., 2014). Therefore, these *in vivo* effects can be directly related to the improved pharmacological profile at α2$GABA_ARs$. Thus HZ-166 might also be a good candidate for the study of BDZs as a therapeutic approach against chronic pain in humans.

We also evaluated the modulatory activities of two novel BDZs, namely TP003 and TPA023B. TP003 has been shown to have anxiolytic effects *in vivo*, and acting specifically at α3$GABA_ARs$ (Dias et al., 2005). However, conflicting evidence of its selectivity to α3$GABA_ARs$ has been recently demonstrated (de Lucas et al., 2015). Therefore, in-house analysis of the modulatory effect of TP003 was required to develop further behavioral experiments in animal models. We have found that TP003 modulates all four $GABA_AR$ combinations, showing higher potency and efficacy at α3$GABA_ARs$. These results are in line with what has been published previously (de Lucas et al., 2015). The discrepancy with Dias and colleagues (2005) might come from the different expression system used (stably expressed mouse fibroblast L(tk^-) cell lines in Dias et al., 2005). Dias and colleagues also

reported that binding affinities of TP003 at α1, α2, α3 and α5$GABA_A$Rs are comparable (Dias et al., 2005), indicating that TP003 binds to the benzodiazepine-binding site and eventually exerts modulatory efficacy.

TPA023B, a novel BDZ that has progressed to clinical trials (Russell et al., 2006), showed the strongest selectivity profile, with the best α2/α1 selectivity ratio and highest efficacy at α2 and α3$GABA_A$Rs. These results are in agreement with previous findings (Van Laere et al., 2008, Atack et al., 2011). It has been previously shown that TPA023B shows a favorable pharmacokinetic profile. In phase I clinical trials it demonstrated a PK/PD profile suitable for dosing in human (Russell et al., 2006).

Pharmacological enhancement of GABAergic synaptic inhibition in the spinal dorsal horn should have a beneficial effect on chronic pain syndromes that result from reduced presynaptic GABA release or from reduced responsiveness of postsynaptic $GABA_A$Rs. However, one question arises on whether a potentiation of GABAergic responses will restore proper inhibition when glycinergic neurotransmission is also compromised. Most of inhibitory interneurons of the dorsal spinal cord co-release GABA and glycine (Todd and Sullivan, 1990, Bohlhalter et al., 1996, Todd et al., 1996, Colin et al., 1998, Feng and Durand, 2005). Moreover, in cells in which no GABAergic component is noticeable under normal conditions, a GABAergic element can be revealed with benzodiazepines and neurosteroids (Keller et al., 2001, Keller et al., 2004). Therefore, it is conceivable that pharmacological modulation of GABAergic transmission would also compensate for the reduction of glycinergic neurotransmission.

It is well established that classical BDZs require the presence of α subunits (α1-3, 5) and the γ2 subunit for full potentiation of $GABA_A$Rs. However, γ1-containing $GABA_A$Rs still show sensitivity to certain BDZs (McKernan et al., 1995, Esmaeili et al., 2009). The γ1 subunit is rather restricted to certain brain nuclei, whereas the γ2 subunit is expressed throughout the brain with high levels in the cortex (Hortnagl et al., 2013). Current data from the Gensat Project provides evidence of the expression of the γ1 subunit in the spinal cord. It is thus necessary to confirm these data performing in-house Taq-man and/or immunohistochemical analysis. Enhancing the activity of $GABA_A$Rs that express the γ1 subunit might avoid many supraspinally mediated BDZ effects and provide an extra level of specificity. I therefore aimed to characterize several novel BDZs in which we could find an increase of potency and/or efficacy targeting the α2 subunit (relevant for spinal-mediated analgesia) co-expressed with the γ1 subunit in the $GABA_A$R complex. Our data suggest that the best candidate was HZ-166 with efficacy values over 380%. The modulation of HZ-166 at α2β3γ1$GABA_A$Rs was dependent on the BDZ-binding site at α subunit, and required the expression of the γ1 subunit. Additionally, HZ-166 showed reduced activity at

α1β2γ1GABA$_A$Rs. However, the exchange of α2 to α3 in the receptor complex did not change the efficacy over the range of concentration tested.

Taken together, these results support the effort of searching for new positive allosteric modulation acting preferentially at the α2/γ1 interface, which in turn will lead to an additional level of selectivity for analgesia at the spinal level, avoiding unwanted supraspinal effects. In addition, the data on HZ-166 show that this compound will be a useful tool to study a potential role of γ1-containing GABA$_A$Rs in GABA mediated spinal analgesia.

REFERENCES

Ahmadi, S., S. Lippross, W. L. Neuhuber and H. U. Zeilhofer (2002). "PGE(2) selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons." Nature neuroscience **5**(1): 34-40.

Atack, J. R., D. J. Hallett, S. Tye, K. A. Wafford, C. Ryan, S. M. Sanabria-Bohorquez, W. S. Eng, R. E. Gibson, H. D. Burns, G. R. Dawson, R. W. Carling, L. J. Street, A. Pike, I. De Lepeleire, K. Van Laere, G. Bormans, J. N. de Hoon, A. Van Hecken, R. M. McKernan, M. G. Murphy and R. J. Hargreaves (2011). "Preclinical and clinical pharmacology of TPA023B, a GABAA receptor α2/α3 subtype-selective partial agonist." Journal of psychopharmacology **25**(3): 329-344.

Besson, M., Y. Daali, A. Di Lio, P. Dayer, H. U. Zeilhofer and J. Desmeules (2013). "Antihyperalgesic effect of the GABA(A) ligand clobazam in a neuropathic pain model in mice: a pharmacokinetic-pharmacodynamic study." Basic & clinical pharmacology & toxicology **112**(3): 192-197.

Bohlhalter, S., O. Weinmann, H. Mohler and J. M. Fritschy (1996). "Laminar compartmentalization of GABAA-receptor subtypes in the spinal cord: an immunohistochemical study." The Journal of neuroscience : the official journal of the Society for Neuroscience **16**(1): 283-297.

Colin, I., P. Rostaing, A. Augustin and A. Triller (1998). "Localization of components of glycinergic synapses during rat spinal cord development." The Journal of comparative neurology **398**(3): 359-372.

Coull, J. A., S. Beggs, D. Boudreau, D. Boivin, M. Tsuda, K. Inoue, C. Gravel, M. W. Salter and Y. De Koninck (2005). "BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain." Nature **438**(7070): 1017-1021.

Coull, J. A., D. Boudreau, K. Bachand, S. A. Prescott, F. Nault, A. Sik, P. De Koninck and Y. De Koninck (2003). "Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain." Nature **424**(6951): 938-942.

de Lucas, A. G., P. K. Ahring, J. S. Larsen, I. Rivera-Arconada, J. A. Lopez-Garcia, N. R. Mirza and G. Munro (2015). "GABAA alpha5 subunit-containing receptors do not contribute to reversal of inflammatory-induced spinal sensitization as indicated by the unique selectivity profile of the GABAA receptor allosteric modulator NS16085." Biochemical pharmacology **93**(3): 370-379.

Di Lio, A., D. Benke, M. Besson, J. Desmeules, Y. Daali, Z. J. Wang, R. Edwankar, J. M. Cook and H. U. Zeilhofer (2011). "HZ166, a novel GABAA receptor subtype-selective benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain." Neuropharmacology **60**(4): 626-632.

Dias, R., W. F. Sheppard, R. L. Fradley, E. M. Garrett, J. L. Stanley, S. J. Tye, S. Goodacre, R. J. Lincoln, S. M. Cook, R. Conley, D. Hallett, A. C. Humphries, S. A. Thompson, K. A. Wafford, L. J. Street, J. L. Castro, P. J. Whiting, T. W. Rosahl, J. R. Atack, R. M. McKernan, G. R. Dawson and D. S. Reynolds (2005). "Evidence for a significant role of alpha 3-containing GABAA receptors in mediating the anxiolytic effects of benzodiazepines." The Journal of neuroscience : the official journal of the Society for Neuroscience **25**(46): 10682-10688.

Draguhn, A., T. A. Verdorn, M. Ewert, P. H. Seeburg and B. Sakmann (1990). "Functional and molecular distinction between recombinant rat GABAA receptor subtypes by Zn2+." Neuron **5**(6): 781-788.

Esmaeili, A., J. W. Lynch and P. Sah (2009). "GABAA receptors containing gamma1 subunits contribute to inhibitory transmission in the central amygdala." Journal of neurophysiology **101**(1): 341-349.

Feng, Z. and D. M. Durand (2005). "Decrease in synaptic transmission can reverse the propagation direction of epileptiform activity in hippocampus *in vivo*." Journal of neurophysiology **93**(3): 1158-1164.

Fritschy, J. M. and I. Brunig (2003). "Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications." Pharmacology & therapeutics **98**(3): 299-323.

Harvey, R. J., U. B. Depner, H. Wassle, S. Ahmadi, C. Heindl, H. Reinold, T. G. Smart, K. Harvey, B. Schutz, O. M. Abo-Salem, A. Zimmer, P. Poisbeau, H. Welzl, D. P. Wolfer, H. Betz, H. U. Zeilhofer and U. Muller (2004). "GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization." Science **304**(5672): 884-887.

Hortnagl, H., R. O. Tasan, A. Wieselthaler, E. Kirchmair, W. Sieghart and G. Sperk (2013). "Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain." Neuroscience **236**: 345-372.

Keller, A. F., S. Beggs, M. W. Salter and Y. De Koninck (2007). "Transformation of the output of spinal lamina I neurons after nerve injury and microglia stimulation underlying neuropathic pain." Molecular pain **3**: 27.

Keller, A. F., J. D. Breton, R. Schlichter and P. Poisbeau (2004). "Production of 5alpha-reduced neurosteroids is developmentally regulated and shapes GABA(A) miniature IPSCs in lamina II of the spinal cord." The Journal of neuroscience : the official journal of the Society for Neuroscience **24**(4): 907-915.

Keller, A. F., J. A. Coull, N. Chery, P. Poisbeau and Y. De Koninck (2001). "Region-specific developmental specialization of GABA-glycine cosynapses in laminas I-II of the rat spinal dorsal horn." The Journal of neuroscience : the official journal of the Society for Neuroscience **21**(20): 7871-7880.

Khom, S., I. Baburin, E. N. Timin, A. Hohaus, W. Sieghart and S. Hering (2006). "Pharmacological properties of GABAA receptors containing gamma1 subunits." Molecular pharmacology **69**(2): 640-649.

Knabl, J., R. Witschi, K. Hosl, H. Reinold, U. B. Zeilhofer, S. Ahmadi, J. Brockhaus, M. Sergejeva, A. Hess, K. Brune, J. M. Fritschy, U. Rudolph, H. Mohler and H. U. Zeilhofer (2008). "Reversal of pathological pain through specific spinal GABAA receptor subtypes." Nature **451**(7176): 330-334.

McKernan, R. M., K. Wafford, K. Quirk, K. L. Hadingham, E. A. Harley, C. I. Ragan and P. J. Whiting (1995). "The pharmacology of the benzodiazepine site of the GABA-A receptor is dependent on the type of gamma-subunit present." Journal of receptor and signal transduction research **15**(1-4): 173-183.

Mohler, H., J. M. Fritschy and U. Rudolph (2002). "A new benzodiazepine pharmacology." The Journal of pharmacology and experimental therapeutics **300**(1): 2-8.

Moore, K. A., T. Kohno, L. A. Karchewski, J. Scholz, H. Baba and C. J. Woolf (2002). "Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord." The Journal of neuroscience : the official journal of the Society for Neuroscience **22**(15): 6724-6731.

Munro, G., H. K. Erichsen, M. G. Rae and N. R. Mirza (2011). "A question of balance--positive versus negative allosteric modulation of GABA(A) receptor subtypes as a driver of analgesic efficacy in rat models of inflammatory and neuropathic pain." Neuropharmacology **61**(1-2): 121-132.

Paul, J., G. E. Yevenes, D. Benke, A. Di Lio, W. T. Ralvenius, R. Witschi, L. Scheurer, J. M. Cook, U. Rudolph, J. M. Fritschy and H. U. Zeilhofer (2014). "Antihyperalgesia by alpha2-GABAA receptors occurs via a genuine spinal action and does not involve supraspinal sites." Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology **39**(2): 477-487.

Pernia-Andrade, A. J., A. Kato, R. Witschi, R. Nyilas, I. Katona, T. F. Freund, M. Watanabe, J. Filitz, W. Koppert, J. Schuttler, G. Ji, V. Neugebauer, G. Marsicano, B. Lutz, H. Vanegas and H. U. Zeilhofer (2009). "Spinal endocannabinoids and CB1 receptors mediate C-fiber-induced heterosynaptic pain sensitization." Science **325**(5941): 760-764.

Ralvenius, W. T., M. A. Acuna, D. Benke, A. Matthey, Y. Daali, U. Rudolph, J. Desmeules, H. U. Zeilhofer and M. Besson (2016). "The clobazam metabolite N-desmethyl clobazam

is an alpha2 preferring benzodiazepine with an improved therapeutic window for antihyperalgesia." Neuropharmacology **109**: 366-375.

Ralvenius, W. T., D. Benke, M. A. Acuna, U. Rudolph and H. U. Zeilhofer (2015). "Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABAA receptor subtype." Nature communications **6**: 6803.

Rivas, F. M., J. P. Stables, L. Murphree, R. V. Edwankar, C. R. Edwankar, S. Huang, H. D. Jain, H. Zhou, S. Majumder, S. Sankar, B. L. Roth, J. Ramerstorfer, R. Furtmuller, W. Sieghart and J. M. Cook (2009). "Antiseizure activity of novel gamma-aminobutyric acid (A) receptor subtype-selective benzodiazepine analogues in mice and rat models." Journal of medicinal chemistry **52**(7): 1795-1798.

Russell, M. G., R. W. Carling, L. J. Street, D. J. Hallett, S. Goodacre, E. Mezzogori, M. Reader, S. M. Cook, F. A. Bromidge, R. Newman, A. J. Smith, K. A. Wafford, G. R. Marshall, D. S. Reynolds, R. Dias, P. Ferris, J. Stanley, R. Lincoln, S. J. Tye, W. F. Sheppard, B. Sohal, A. Pike, M. Dominguez, J. R. Atack and J. L. Castro (2006). "Discovery of imidazo[1,2-b][1,2,4]triazines as GABA(A) alpha2/3 subtype selective agonists for the treatment of anxiety." Journal of medicinal chemistry **49**(4): 1235-1238.

Scott-Stevens, P., J. R. Atack, B. Sohal and P. Worboys (2005). "Rodent pharmacokinetics and receptor occupancy of the GABAA receptor subtype selective benzodiazepine site ligand L-838417." Biopharmaceutics & drug disposition **26**(1): 13-20.

Smart, T. G., S. J. Moss, X. Xie and R. L. Huganir (1991). "GABAA receptors are differentially sensitive to zinc: dependence on subunit composition." British journal of pharmacology **103**(4): 1837-1839.

Todd, A. J. and A. C. Sullivan (1990). "Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat." The Journal of comparative neurology **296**(3): 496-505.

Todd, A. J., C. Watt, R. C. Spike and W. Sieghart (1996). "Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord." The Journal of neuroscience : the official journal of the Society for Neuroscience **16**(3): 974-982.

Van Laere, K., G. Bormans, S. M. Sanabria-Bohorquez, T. de Groot, P. Dupont, I. De Lepeleire, J. de Hoon, L. Mortelmans, R. J. Hargreaves, J. R. Atack and H. D. Burns (2008). "*In vivo* characterization and dynamic receptor occupancy imaging of TPA023B, an alpha 2/alpha 3/alpha 5 subtype selective gamma-aminobutyric acid-a partial agonist." Biological psychiatry **64**(2): 153-161.

Wieland, H. A., H. Luddens and P. H. Seeburg (1992). "Molecular determinants in GABAA/BZ receptor subtypes." Advances in biochemical psychopharmacology **47**: 29-40.

Witschi, R., P. Punnakkal, J. Paul, J. S. Walczak, F. Cervero, J. M. Fritschy, R. Kuner, R. Keist, U. Rudolph and H. U. Zeilhofer (2011). "Presynaptic alpha2-GABAA receptors in primary afferent depolarization and spinal pain control." J Neurosci **31**(22): 8134-8142.

Zhang, Z., Y. Q. Cai, F. Zou, B. Bie and Z. Z. Pan (2011). "Epigenetic suppression of GAD65 expression mediates persistent pain." Nature medicine **17**(11): 1448-1455.

4. General Discussion and Out-Look

In pharmacology, the therapeutic window refers to a dose range in which there are few or no side effects. Adding degrees of selectivity of a drug for a specific target, it is then conceivable to increase the therapeutic window. Therefore, the main goal of this thesis was to identify compounds and targets that allow a specific or at least preferential action on the nociceptive system.

The results summarized in **Chapter I** provide evidence for the first time that a high level of specificity can be achieved by targeting synaptic glycine receptors in the phosphorylated state, which are key contributors to spinal inflammatory pain sensitization. Un-primed recombinant α3/β GlyRs are not sensitive to the test compound 2,6-di-*tert*-butylphenol (2,6-DTBP). However, by exchanging the serine in the PKA consensus phosphorylation site (S346) to a negatively charged amino acid (S346E), α3(S346E)βGlyRs became sensitive to modulation by 2,6-DTBP. Furthermore, we confirmed that synaptic (presumable α3/β) GlyRs are sensitive to 2,6-DTBP only after exposure to PGE_2, which leads to PKA dependent phosphorylation of GlyRα3. Moreover, mice treated with 2,6-DTBP did not develop impaired locomotor activity, motor coordination or muscle strength. Strikingly, 2,6-DTBP showed anti-hyperalgesic effects in inflammatory pain models. These effects were significantly decreased in mice lacking the GlyRα3 subunit, indicating a central role of α3GlyR in the anti-hyperalgesic actions of 2,6-DTBP.

Post-translational modifications – such as phosphorylation – can promote a global conformational change of pentameric ligand-gated ion channels. Phosphorylation-induced conformational changes in the α3GlyR have been recently reported using voltage clamp fluorometry (Han et al., 2013). This technique is based on changes in the quantum efficiency of fluorophores covalently bound to a cysteine introduced into the domain of interest. It has been shown that phosphorylation at S346 of the GlyRα3 subunit promotes a change of fluorescence in the signal when the fluorophore is linked to the glycine-binding site (Han et al., 2013). These results indicate that a conformational change propagates from the M3-M4 intracellular loop to the agonist- binding site located at about 80Å. Therefore, it is tempting to speculate that, if phosphorylation alters the structure of orthosteric and/or allosteric binding sites, then binding of a drug to a phosphorylated-“primed” receptor may compensate for the effects of the phosphorylation (Talwar and Lynch, 2014).

During the last two years there have been increasing efforts to develop new compounds targeting GlyRs (Eckle et al., 2014, Farley and Mihic, 2015, Maleeva et al., 2015b, a, Shalaly et al., 2015, Breitinger and Breitinger, 2016, Breitinger et al., 2016, Cornelison et al., 2016,

Germann et al., 2016, Lara et al., 2016, Yin et al., 2016). Moreover, researchers at Neusentis (Granta Park, Great Abington, Cambridgeshire, UK) have validated the use of a high-throughput fluorescent membrane potential screening for the development of new positive allosteric modulators of α3GlyRs as novel analgesic (Stead et al., 2016). These techniques will help the functional characterization of compounds with high efficacy and potency acting at GlyRs in an efficient manner. However, despite the fact that using high-throughput screening leads to the identification of hit compounds much faster than conventional approaches, the screening operation also gives low genuine hit rate and a large number of false positives (Jensen and Kristiansen, 2004, Ghisdal et al., 2014). This point encourages the use of conventional techniques, such as manual patch-clamp, to verify possible true candidates. The work of the Neusentis team has shown that one compound (compound Nr. 2 in (Stead et al., 2016)), which was tested at homomeric GlyRs, shows high efficacy at α3GlyR and α1GlyRs in the absence of activity at $GABA_A$Rs (α1β2γ2). It is at present unknown whether this compound exerts analgesic effects *in vivo*.

A relevant question is whether the enhancement of spinal inhibitory neurotransmission has a real impact on pain treatment in humans. The α3 subunit of the GlyR has two isoforms in human. Both isoforms, named “a” and “b” (RefSeq: NP_001036008.1 and NP_006520.2, respectively), have a consensus sequence for recognition and phosphorylation by PKA at the serine residue (Arg-Glu-**Ser**-Arg). Although, to our knowledge, there is no information in human tissue of an inhibition of α3GlyRs upon activation of EP2 receptor, the mechanism underlying a PGE_2-mediated sensitization may be the same as in mice. Moreover, GlyR distribution in human tissue is similar to the distribution in the rodent spinal cord, as judged by immunostaining post-mortem tissue (reviewed in Baer et al., 2009). Therefore, targeting specifically those inflammation-primed GlyRs in humans might be a promising target to alleviate inflammatory pain.

Our data provide evidence on the molecular determinants for binding of 2,6-DTBP at the GlyRα3. We identify that the phenylalanine at the position 388 (F388) is required for modulatory actions of 2,6-DTBP at homomeric α3GlyRs. This residue is also necessary for the modulation of propofol at homomeric α1GlyRs (Moraga-Cid et al., 2011). Potentiation of propofol at $GABA_A$Rs occurs with about 8-times higher potency (Pistis et al., 1997) than that of homomeric α1GlyRs and α3GlyRs. This low potency displayed in our *in vitro* studies reflects the high doses of 2,6-DTBP used to achieve antihyperalgesic effects *in vivo*. Furthermore, 2,6-DTBP evoked analgesia not only through α3GlyRs but also through other targets. Therefore, in order to test whether our findings in mouse pain models are relevant to human pain condition, compounds need to be developed with increased potency at phosphorylated a3GlyR and reduced off-target effects.

Phosphorylation of the GlyRα3 subunit does not only occur in inflammatory pain states. Instead, it also controls rhythmic breathing (Manzke et al., 2010). Serotonin induces a de-phosphorylation of α3GlyRs at S346 – thereby enhancing glycinergic activity – through an activation of the serotonin receptor 5-HTR_{1A}. Stimulation of this pathway reverses opioid-induced respiratory depression, suggesting that enhancing the activity of phosphorylated α3GlyR might remedy this respiratory alteration. Therefore, it is tempting to speculate that a selective potentiation of phosphorylated-primed α3GlyRs might not only restore inflammatory mediated spinal disinhibition, but also could affect some others mechanisms involving the phosphorylation and eventual reduction of glycinergic inhibition.

Can a high level of specificity be reached targeting $GABA_ARs$?

The results in **Chapter II** provide evidence that certain benzodiazepine site agonists or partial agonists (namely NDMC, HZ-166 and TPA023B) show a more favourable α2 over α1 activity. Pre-clinical studies suggest that such compounds should be well-suited for the treatment of persistent pain (Knabl et al., 2008, Di Lio et al., 2011, Paul et al., 2014, Ralvenius et al., 2015). To increase the level of specificity of a potential drug, we decided to explore of the γ subunit subtype as an additional way to increase specificity. In the brain, the γ1-subunit is expressed only in very low levels whereas some data from public website and from our group suggest a higher expression level in the dorsal horn. I had therefore decided to determine the efficacy at γ1 $GABA_ARs$ of selected BDZ site ligands with a favourable α2/α1 or analgesia/sedation ratio. Among the compounds tested HZ-166 had the most promising γ1/γ2 activity ratio α2$GABA_ARs$. On-going experiments in our group use this knowledge to test γ1 containing $GABA_ARs$ make a relevant contribution to spinal analgesia by HZ-166. A reasonable way to test whether HZ-166 exerts an *in vivo* effect at γ1$GABA_ARs$ is to knock-out the γ2 subunit at spinal level. This can be done taking advantage of the Hoxb8-cre mouse line (Witschi et al., 2010), and crossing it with γ2-floxed animals. According to the results described in this thesis, HZ-166 might thus have antihyperalgesic effects, acting at γ1$GABA_ARs$.

A recent publication has shed light on the composition of $GABA_ARs$ in the human spinal cord (Waldvogel et al., 2010). The expression of α1-3 subunits together with β2/3 subunits and the γ2 subunit is similar to levels found in rodents (Paul et al., 2012, Ralvenius et al., 2015). Additionally, recent studies have provided direct evidence for BDZ-mediated analgesia in human volunteers (Vuilleumier et al., 2013, Besson et al., 2015). However, those effects were mild and still accompanied by sedation. In mice, it has been recently described by our group that ED_{50} values (the effective dose at which 50% of the maximal effect is reached) of DZP for sedation and antihyperalgesia are different by a factor of about 20 (Ralvenius et al., 2015). These data indicate that such a difference underlies the dose-limiting sedative effects

of non-selective BDZs. Hence, selectively targeting α2GABA$_A$RS might have the best benefit-risk ratio producing pronounced antihyperalgesic effects in the absence of sedation. Our study in recombinant GABA$_A$Rs has shown that NDMC may have a better profile than a non-selective BDZ. These results translated into *in vivo* improved therapeutic window of NDMC over DZP (Ralvenius et al., 2016). Moreover, using HZ-166 as a pharmacological tool targeting α2 and γ1-containing GABA$_A$Rs, we provide evidence for the first time of an increased level of specificity not only at a higher α2/α1GABA$_A$R ratio, but also at a higher γ1/γ2 ratio.

Most drug discovery efforts in the benzodiazepine field have been into dedicated screening libraries for the development of compounds acting at the canonical benzodiazepine-binding site. These endeavors are conceivable, as γ2-containing receptors form the vast majority of GABA$_A$Rs in the brain and mediate most if not all therapeutic effects of classical benzodiazepines. As shown in this thesis and by others, some of these compounds also act at γ1GABA$_A$Rs albeit with lower potency. To the best of my knowledge, no screening effort has been undertaken specifically targeted at γ1GABA$_A$Rs.

In summary, the results presented in this thesis identified new mechanisms for a specific interaction with inhibitory neurotransmitter receptors involved in the spinal control of pain.

5. References

Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU (2002) PGE(2) selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci 5:34-40.

Ahrens J, Haeseler G, Leuwer M, Mohammadi B, Krampfl K, Dengler R, Bufler J (2004) 2,6 di-tert-butylphenol, a nonanesthetic propofol analog, modulates alpha1beta glycine receptor function in a manner distinct from propofol. Anesth Analg 99:91-96.

Ahrens J, Leuwer M, Demir R, Krampfl K, de la Roche J, Foadi N, Karst M, Haeseler G (2009) Positive allosteric modulatory effects of ajulemic acid at strychnine-sensitive glycine alpha1- and alpha1beta-receptors. Naunyn Schmiedebergs Arch Pharmacol 379:371-378.

Apkarian AV (2013) A brain signature for acute pain. Trends Cogn Sci 17:309-310.

Araki T, Yamano M, Murakami T, Wanaka A, Betz H, Tohyama M (1988) Localization of glycine receptors in the rat central nervous system: an immunocytochemical analysis using monoclonal antibody. Neuroscience 25:613-624.

Atack JR, Hallett DJ, Tye S, Wafford KA, Ryan C, Sanabria-Bohorquez SM, Eng WS, Gibson RE, Burns HD, Dawson GR, Carling RW, Street LJ, Pike A, De Lepeleire I, Van Laere K, Bormans G, de Hoon JN, Van Hecken A, McKernan RM, Murphy MG, Hargreaves RJ (2011) Preclinical and clinical pharmacology of TPA023B, a GABAA receptor α2/α3 subtype-selective partial agonist. J Psychopharmacol 25:329-344.

Auvray M, Myin E, Spence C (2010) The sensory-discriminative and affective-motivational aspects of pain. Neurosci Biobehav Rev 34:214-223.

Baburin I, Khom S, Timin E, Hohaus A, Sieghart W, Hering S (2008) Estimating the efficiency of benzodiazepines on GABA(A) receptors comprising gamma1 or gamma2 subunits. Br J Pharmacol 155:424-433.

Baer K, Waldvogel HJ, Faull RL, Rees MI (2009) Localization of glycine receptors in the human forebrain, brainstem, and cervical spinal cord: an immunohistochemical review. Front Mol Neurosci 2:25.

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. Cell 139:267-284.

Beato M, Sivilotti LG (2007) Single-channel properties of glycine receptors of juvenile rat spinal motoneurones in vitro. J Physiol 580:497-506.

Becker J, Li Z, Noe CR (1998) Molecular and pharmacological characterization of recombinant rat/mice N-methyl-D-aspartate receptor subtypes in the yeast Saccharomyces cerevisiae. Eur J Biochem 256:427-435.

Belelli D, Harrison NL, Maguire J, Macdonald RL, Walker MC, Cope DW (2009) Extrasynaptic GABAA receptors: form, pharmacology, and function. J Neurosci 29:12757-12763.

Belelli D, Pistis M, Peters JA, Lambert JJ (1999) General anaesthetic action at transmitter-gated inhibitory amino acid receptors. Trends Pharmacol Sci 20:496-502.

Benke D, Honer M, Michel C, Mohler H (1996) GABAA receptor subtypes differentiated by their gamma-subunit variants: prevalence, pharmacology and subunit architecture. Neuropharmacology 35:1413-1423.

Besson M, Daali Y, Di Lio A, Dayer P, Zeilhofer HU, Desmeules J (2013) Antihyperalgesic effect of the GABA(A) ligand clobazam in a neuropathic pain model in mice: a pharmacokinetic-pharmacodynamic study. Basic Clin Pharmacol Toxicol 112:192-197.

Besson M, Matthey A, Daali Y, Poncet A, Vuilleumier P, Curatolo M, Zeilhofer HU, Desmeules J (2015) GABAergic modulation in central sensitization in humans: a randomized placebo-controlled pharmacokinetic-pharmacodynamic study comparing clobazam with clonazepam in healthy volunteers. Pain 156:397-404.

Beyer C, Roberts LA, Komisaruk BR (1985) Hyperalgesia induced by altered glycinergic activity at the spinal cord. Life Sci 37:875-882.
Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol 232:331-356.
Bogen O, Joseph EK, Chen X, Levine JD (2008) GDNF hyperalgesia is mediated by PLCgamma, MAPK/ERK, PI3K, CDK5 and Src family kinase signaling and dependent on the IB4-binding protein versican. Eur J Neurosci 28:12-19.
Bohlhalter S, Weinmann O, Mohler H, Fritschy JM (1996) Laminar compartmentalization of GABAA-receptor subtypes in the spinal cord: an immunohistochemical study. J Neurosci 16:283-297.
Bonica JJ (1979) The need of a taxonomy. Pain 6:247-248.
Braz JM, Nassar MA, Wood JN, Basbaum AI (2005) Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. Neuron 47:787-793.
Breitinger U, Breitinger HG (2016) Augmentation of glycine receptor alpha3 currents suggests a mechanism for glucose-mediated analgesia. Neurosci Lett 612:110-115.
Breitinger U, Sticht H, Breitinger HG (2016) Modulation of Recombinant Human alpha1 Glycine Receptors by Mono- and Disaccharides: A Kinetic Study. ACS Chem Neurosci 7:1077-1087.
Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D (2006) Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. Eur J Pain 10:287-333.
Burzomato V, Beato M, Groot-Kormelink PJ, Colquhoun D, Sivilotti LG (2004) Single-channel behavior of heteromeric alpha1beta glycine receptors: an attempt to detect a conformational change before the channel opens. J Neurosci 24:10924-10940.
Colin I, Rostaing P, Augustin A, Triller A (1998) Localization of components of glycinergic synapses during rat spinal cord development. J Comp Neurol 398:359-372.
Cope DW, Halbsguth C, Karayannis T, Wulff P, Ferraguti F, Hoeger H, Leppa E, Linden AM, Oberto A, Ogris W, Korpi ER, Sieghart W, Somogyi P, Wisden W, Capogna M (2005) Loss of zolpidem efficacy in the hippocampus of mice with the GABAA receptor gamma2 F77I point mutation. Eur J Neurosci 21:3002-3016.
Cope DW, Wulff P, Oberto A, Aller MI, Capogna M, Ferraguti F, Halbsguth C, Hoeger H, Jolin HE, Jones A, McKenzie AN, Ogris W, Poeltl A, Sinkkonen ST, Vekovischeva OY, Korpi ER, Sieghart W, Sigel E, Somogyi P, Wisden W (2004) Abolition of zolpidem sensitivity in mice with a point mutation in the GABAA receptor gamma2 subunit. Neuropharmacology 47:17-34.
Cornelison GL, Pflanz NC, Tipps ME, Mihic SJ (2016) Identification and characterization of heptapeptide modulators of the glycine receptor. Eur J Pharmacol 780:252-259.
Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y (2005) BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438:1017-1021.
Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De Koninck P, De Koninck Y (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 424:938-942.
Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG (2006) An SCN9A channelopathy causes congenital inability to experience pain. Nature 444:894-898.
Craig AD (2003) Pain mechanisms: labeled lines versus convergence in central processing. Annu Rev Neurosci 26:1-30.
de Lucas AG, Ahring PK, Larsen JS, Rivera-Arconada I, Lopez-Garcia JA, Mirza NR, Munro G (2015) GABAA alpha5 subunit-containing receptors do not contribute to reversal of inflammatory-induced spinal sensitization as indicated by the unique selectivity profile of the GABAA receptor allosteric modulator NS16085. Biochem Pharmacol 93:370-379.

Deleuze C, Alonso G, Lefevre IA, Duvoid-Guillou A, Hussy N (2005) Extrasynaptic localization of glycine receptors in the rat supraoptic nucleus: further evidence for their involvement in glia-to-neuron communication. Neuroscience 133:175-183.

Di Lio A, Benke D, Besson M, Desmeules J, Daali Y, Wang ZJ, Edwankar R, Cook JM, Zeilhofer HU (2011) HZ166, a novel GABAA receptor subtype-selective benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain. Neuropharmacology 60:626-632.

Dias R, Sheppard WF, Fradley RL, Garrett EM, Stanley JL, Tye SJ, Goodacre S, Lincoln RJ, Cook SM, Conley R, Hallett D, Humphries AC, Thompson SA, Wafford KA, Street LJ, Castro JL, Whiting PJ, Rosahl TW, Atack JR, McKernan RM, Dawson GR, Reynolds DS (2005) Evidence for a significant role of alpha 3-containing GABAA receptors in mediating the anxiolytic effects of benzodiazepines. J Neurosci 25:10682-10688.

Downie DL, Hall AC, Lieb WR, Franks NP (1996) Effects of inhalational general anaesthetics on native glycine receptors in rat medullary neurones and recombinant glycine receptors in Xenopus oocytes. Br J Pharmacol 118:493-502.

Draguhn A, Verdorn TA, Ewert M, Seeburg PH, Sakmann B (1990) Functional and molecular distinction between recombinant rat GABAA receptor subtypes by Zn2+. Neuron 5:781-788.

Eckle VS, Grasshoff C, Mirakaj V, O'Neill PM, Berry NG, Leuwer M, Antkowiak B (2014) 4-bromopropofol decreases action potential generation in spinal neurons by inducing a glycine receptor-mediated tonic conductance. Br J Pharmacol 171:5790-5801.

Esmaeili A, Lynch JW, Sah P (2009) GABAA receptors containing gamma1 subunits contribute to inhibitory transmission in the central amygdala. J Neurophysiol 101:341-349.

Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin. Nat Neurosci 1:563-571.

Farley NM, Mihic SJ (2015) Allosteric modulation of the glycine receptor activated by agonists differing in efficacy. Brain Res 1606:95-101.

Feng Z, Durand DM (2005) Decrease in synaptic transmission can reverse the propagation direction of epileptiform activity in hippocampus in vivo. J Neurophysiol 93:1158-1164.

Fields HL, Heinricher MM (1985) Anatomy and physiology of a nociceptive modulatory system. Philos Trans R Soc Lond B Biol Sci 308:361-374.

Fields HL, Heinricher MM, Mason P (1991) Neurotransmitters in nociceptive modulatory circuits. Annu Rev Neurosci 14:219-245.

Fjell J, Cummins TR, Fried K, Black JA, Waxman SG (1999) In vivo NGF deprivation reduces SNS expression and TTX-R sodium currents in IB4-negative DRG neurons. J Neurophysiol 81:803-810.

Foster E, Wildner H, Tudeau L, Haueter S, Ralvenius WT, Jegen M, Johannssen H, Hosli L, Haenraets K, Ghanem A, Conzelmann KK, Bosl M, Zeilhofer HU (2015) Targeted ablation, silencing, and activation establish glycinergic dorsal horn neurons as key components of a spinal gate for pain and itch. Neuron 85:1289-1304.

Fritschy JM, Brunig I (2003) Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. Pharmacol Ther 98:299-323.

Fritschy JM, Harvey RJ, Schwarz G (2008) Gephyrin: where do we stand, where do we go? Trends Neurosci 31:257-264.

Gao YJ, Ren WH, Zhang YQ, Zhao ZQ (2004) Contributions of the anterior cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats. Pain 110:343-353.

Gauriau C, Bernard JF (2004) Posterior triangular thalamic neurons convey nociceptive messages to the secondary somatosensory and insular cortices in the rat. J Neurosci 24:752-761.

Gebhart GF (2004) Descending modulation of pain. Neurosci Biobehav Rev 27:729-737.

Germann AL, Shin DJ, Manion BD, Edge CJ, Smith EH, Franks NP, Evers AS, Akk G (2016) Activation and modulation of recombinant glycine and GABAA receptors by 4-halogenated analogues of propofol. Br J Pharmacol.

Ghisdal P, Noel N, Pacico N, Martini M, Foerch P, Hanon E, Wolff C (2014) Determining the relative efficacy of positive allosteric modulators of the GABAA receptor: design of a screening approach. J Biomol Screen 19:462-467.

Grasshoff C, Antkowiak B (2004) Propofol and sevoflurane depress spinal neurons in vitro via different molecular targets. Anesthesiology 101:1167-1176.

Grudzinska J, Schemm R, Haeger S, Nicke A, Schmalzing G, Betz H, Laube B (2005) The beta subunit determines the ligand binding properties of synaptic glycine receptors. Neuron 45:727-739.

Han L, Talwar S, Wang Q, Shan Q, Lynch JW (2013) Phosphorylation of alpha3 glycine receptors induces a conformational change in the glycine-binding site. ACS Chem Neurosci 4:1361-1370.

Harrison NL, Kugler JL, Jones MV, Greenblatt EP, Pritchett DB (1993) Positive modulation of human gamma-aminobutyric acid type A and glycine receptors by the inhalation anesthetic isoflurane. Mol Pharmacol 44:628-632.

Harvey RJ, Depner UB, Wassle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schutz B, Abo-Salem OM, Zimmer A, Poisbeau P, Welzl H, Wolfer DP, Betz H, Zeilhofer HU, Muller U (2004) GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. Science 304:884-887.

Hejazi N, Zhou C, Oz M, Sun H, Ye JH, Zhang L (2006) Delta9-tetrahydrocannabinol and endogenous cannabinoid anandamide directly potentiate the function of glycine receptors. Mol Pharmacol 69:991-997.

Hofmann M, Kordas KS, Gravius A, Bolcskei K, Parsons CG, Dekundy A, Danysz W, Dezsi L, Wittko-Schneider IM, Saghy K, Gyertyan I, Horvath C (2012) Assessment of the effects of NS11394 and L-838417, alpha2/3 subunit-selective GABA(A) [corrected] receptor-positive allosteric modulators, in tests for pain, anxiety, memory and motor function. Behav Pharmacol 23:790-801.

Hortnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G (2013) Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. Neuroscience 236:345-372.

Ikeda H, Heinke B, Ruscheweyh R, Sandkuhler J (2003) Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science 299:1237-1240.

Jensen AA, Kristiansen U (2004) Functional characterisation of the human alpha1 glycine receptor in a fluorescence-based membrane potential assay. Biochem Pharmacol 67:1789-1799.

Jeong HJ, Jang IS, Moorhouse AJ, Akaike N (2003) Activation of presynaptic glycine receptors facilitates glycine release from presynaptic terminals synapsing onto rat spinal sacral dorsal commissural nucleus neurons. J Physiol 550:373-383.

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203-210.

Keller AF, Beggs S, Salter MW, De Koninck Y (2007) Transformation of the output of spinal lamina I neurons after nerve injury and microglia stimulation underlying neuropathic pain. Mol Pain 3:27.

Keller AF, Breton JD, Schlichter R, Poisbeau P (2004) Production of 5alpha-reduced neurosteroids is developmentally regulated and shapes GABA(A) miniature IPSCs in lamina II of the spinal cord. J Neurosci 24:907-915.

Keller AF, Coull JA, Chery N, Poisbeau P, De Koninck Y (2001) Region-specific developmental specialization of GABA-glycine cosynapses in laminas I-II of the rat spinal dorsal horn. J Neurosci 21:7871-7880.

Khom S, Baburin I, Timin EN, Hohaus A, Sieghart W, Hering S (2006) Pharmacological properties of GABAA receptors containing gamma1 subunits. Mol Pharmacol 69:640-649.

Knabl J, Witschi R, Hosl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Mohler H, Zeilhofer HU (2008) Reversal of

pathological pain through specific spinal GABAA receptor subtypes. Nature 451:330-334.
Kong J, White NS, Kwong KK, Vangel MG, Rosman IS, Gracely RH, Gollub RL (2006) Using fMRI to dissociate sensory encoding from cognitive evaluation of heat pain intensity. Hum Brain Mapp 27:715-721.
Krasowski MD, Harrison NL (1999) General anaesthetic actions on ligand-gated ion channels. Cell Mol Life Sci 55:1278-1303.
Krasowski MD, Jenkins A, Flood P, Kung AY, Hopfinger AJ, Harrison NL (2001) General anesthetic potencies of a series of propofol analogs correlate with potency for potentiation of gamma-aminobutyric acid (GABA) current at the GABA(A) receptor but not with lipid solubility. J Pharmacol Exp Ther 297:338-351.
Kuner R (2010) Central mechanisms of pathological pain. Nat Med 16:1258-1266.
Lara CO, Murath P, Munoz B, Marileo AM, Martin LS, San Martin VP, Burgos CF, Mariqueo TA, Aguayo LG, Fuentealba J, Godoy P, Guzman L, Yevenes GE (2016) Functional modulation of glycine receptors by the alkaloid gelsemine. Br J Pharmacol 173:2263-2277.
Latremoliere A, Woolf CJ (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 10:895-926.
Laube B, Maksay G, Schemm R, Betz H (2002) Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? Trends Pharmacol Sci 23:519-527.
Laurie DJ, Seeburg PH, Wisden W (1992) The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. J Neurosci 12:1063-1076.
Leppa E, Linden AM, Rabe H, Vekovischeva OY, Wulff P, Luddens H, Wisden W, Korpi ER (2011a) Actions of two GABAA receptor benzodiazepine-site ligands that are mediated via non-gamma2-dependent modulation. Eur J Pharmacol 666:111-121.
Leppa E, Linden AM, Vekovischeva OY, Swinny JD, Rantanen V, Toppila E, Hoger H, Sieghart W, Wulff P, Wisden W, Korpi ER (2011b) Removal of GABA(A) receptor gamma2 subunits from parvalbumin neurons causes wide-ranging behavioral alterations. PLoS One 6:e24159.
Leppa E, Vekovischeva OY, Linden AM, Wulff P, Oberto A, Wisden W, Korpi ER (2005) Agonistic effects of the beta-carboline DMCM revealed in GABA(A) receptor gamma 2 subunit F77I point-mutated mice. Neuropharmacology 48:469-478.
Li XY, Ko HG, Chen T, Descalzi G, Koga K, Wang H, Kim SS, Shang Y, Kwak C, Park SW, Shim J, Lee K, Collingridge GL, Kaang BK, Zhuo M (2010) Alleviating neuropathic pain hypersensitivity by inhibiting PKMzeta in the anterior cingulate cortex. Science 330:1400-1404.
Lorenzo LE, Magnussen C, Bailey AL, St Louis M, De Koninck Y, Ribeiro-da-Silva A (2014) Spatial and temporal pattern of changes in the number of GAD65-immunoreactive inhibitory terminals in the rat superficial dorsal horn following peripheral nerve injury. Mol Pain 10:57.
Lozovaya N, Yatsenko N, Beketov A, Tsintsadze T, Burnashev N (2005) Glycine receptors in CNS neurons as a target for nonretrograde action of cannabinoids. J Neurosci 25:7499-7506.
Lynch JW (2004) Molecular structure and function of the glycine receptor chloride channel. Physiol Rev 84:1051-1095.
Lynch JW (2009) Native glycine receptor subtypes and their physiological roles. Neuropharmacology 56:303-309.
Maleeva G, Buldakova S, Bregestovski P (2015a) Ginkgolic acid specifically potentiates alpha 1 glycine receptors. Springerplus 4:L31.
Maleeva G, Buldakova S, Bregestovski P (2015b) Selective potentiation of alpha 1 glycine receptors by ginkgolic acid. Front Mol Neurosci 8:64.
Malin SA, Molliver DC, Koerber HR, Cornuet P, Frye R, Albers KM, Davis BM (2006) Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. J Neurosci 26:8588-8599.

Malosio ML, Marqueze-Pouey B, Kuhse J, Betz H (1991) Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. Embo J 10:2401-2409.
Manzke T, Niebert M, Koch UR, Caley A, Vogelgesang S, Hulsmann S, Ponimaskin E, Muller U, Smart TG, Harvey RJ, Richter DW (2010) Serotonin receptor 1A-modulated phosphorylation of glycine receptor alpha3 controls breathing in mice. J Clin Invest 120:4118-4128.
Markopoulos A (2010) A Handbook of Oral Physiology and Oral Biology: Bentham Science Publishers.
Mascia MP, Machu TK, Harris RA (1996) Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. Br J Pharmacol 119:1331-1336.
May AC, Fleischer W, Kletke O, Haas HL, Sergeeva OA (2013) Benzodiazepine-site pharmacology on GABAA receptors in histaminergic neurons. Br J Pharmacol 170:222-232.
McCoy ES, Taylor-Blake B, Street SE, Pribisko AL, Zheng J, Zylka MJ (2013) Peptidergic CGRPalpha primary sensory neurons encode heat and itch and tonically suppress sensitivity to cold. Neuron 78:138-151.
McKernan RM, Wafford K, Quirk K, Hadingham KL, Harley EA, Ragan CI, Whiting PJ (1995) The pharmacology of the benzodiazepine site of the GABA-A receptor is dependent on the type of gamma-subunit present. J Recept Signal Transduct Res 15:173-183.
Melzack R, Wall PD (1965) Pain mechanisms: a new theory. Science 150:971-979.
Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL (1997) Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. Nature 389:385-389.
Millan MJ (2002) Descending control of pain. Prog Neurobiol 66:355-474.
Mirza NR, Larsen JS, Mathiasen C, Jacobsen TA, Munro G, Erichsen HK, Nielsen AN, Troelsen KB, Nielsen EO, Ahring PK (2008) NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile], a unique subtype-selective GABAA receptor positive allosteric modulator: in vitro actions, pharmacokinetic properties and in vivo anxiolytic efficacy. J Pharmacol Exp Ther 327:954-968.
Mohler H, Crestani F, Rudolph U (2001) GABA(A)-receptor subtypes: a new pharmacology. Curr Opin Pharmacol 1:22-25.
Mohler H, Fritschy JM, Rudolph U (2002) A new benzodiazepine pharmacology. J Pharmacol Exp Ther 300:2-8.
Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci 22:6724-6731.
Moraga-Cid G, Yevenes GE, Schmalzing G, Peoples RW, Aguayo LG (2011) A Single phenylalanine residue in the main intracellular loop of alpha1 gamma-aminobutyric acid type A and glycine receptors influences their sensitivity to propofol. Anesthesiology 115:464-473.
Muller F, Heinke B, Sandkuhler J (2003) Reduction of glycine receptor-mediated miniature inhibitory postsynaptic currents in rat spinal lamina I neurons after peripheral inflammation. Neuroscience 122:799-805.
Munro G, Erichsen HK, Rae MG, Mirza NR (2011) A question of balance--positive versus negative allosteric modulation of GABA(A) receptor subtypes as a driver of analgesic efficacy in rat models of inflammatory and neuropathic pain. Neuropharmacology 61:121-132.
Munro G, Lopez-Garcia JA, Rivera-Arconada I, Erichsen HK, Nielsen EO, Larsen JS, Ahring PK, Mirza NR (2008) Comparison of the novel subtype-selective GABAA receptor-positive allosteric modulator NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile] with diazepam, zolpidem, bretazenil, and gaboxadol in rat models of inflammatory and neuropathic pain. J Pharmacol Exp Ther 327:969-981.

Neumann S, Braz JM, Skinner K, Llewellyn-Smith IJ, Basbaum AI (2008) Innocuous, not noxious, input activates PKCgamma interneurons of the spinal dorsal horn via myelinated afferent fibers. J Neurosci 28:7936-7944.
Nickolls S, Mace H, Fish R, Edye M, Gurrell R, Ivarsson M, Pitcher T, Tanimoto-Mori S, Richardson D, Sweatman C, Nicholson J, Ward C, Jinks J, Bell C, Young K, Rees H, Moss A, Kinloch R, McMurray G (2011) A Comparison of the alpha2/3/5 Selective Positive Allosteric Modulators L-838,417 and TPA023 in Preclinical Models of Inflammatory and Neuropathic Pain. Adv Pharmacol Sci 2011:608912.
Ogris W, Poltl A, Hauer B, Ernst M, Oberto A, Wulff P, Hoger H, Wisden W, Sieghart W (2004) Affinity of various benzodiazepine site ligands in mice with a point mutation in the GABA(A) receptor gamma2 subunit. Biochem Pharmacol 68:1621-1629.
Oz M (2006) Receptor-independent actions of cannabinoids on cell membranes: focus on endocannabinoids. Pharmacol Ther 111:114-144.
Paul J, Yevenes GE, Benke D, Di Lio A, Ralvenius WT, Witschi R, Scheurer L, Cook JM, Rudolph U, Fritschy JM, Zeilhofer HU (2014) Antihyperalgesia by alpha2-GABAA receptors occurs via a genuine spinal action and does not involve supraspinal sites. Neuropsychopharmacology 39:477-487.
Paul J, Zeilhofer HU, Fritschy JM (2012) Selective distribution of GABA(A) receptor subtypes in mouse spinal dorsal horn neurons and primary afferents. J Comp Neurol 520:3895-3911.
Pernia-Andrade AJ, Kato A, Witschi R, Nyilas R, Katona I, Freund TF, Watanabe M, Filitz J, Koppert W, Schuttler J, Ji G, Neugebauer V, Marsicano G, Lutz B, Vanegas H, Zeilhofer HU (2009) Spinal endocannabinoids and CB1 receptors mediate C-fiber-induced heterosynaptic pain sensitization. Science 325:760-764.
Pistis M, Belelli D, Peters JA, Lambert JJ (1997) The interaction of general anaesthetics with recombinant GABAA and glycine receptors expressed in Xenopus laevis oocytes: a comparative study. Br J Pharmacol 122:1707-1719.
Porreca F, Ossipov MH, Gebhart GF (2002) Chronic pain and medullary descending facilitation. Trends Neurosci 25:319-325.
Puia G, Vicini S, Seeburg PH, Costa E (1991) Influence of recombinant gamma-aminobutyric acid-A receptor subunit composition on the action of allosteric modulators of gamma-aminobutyric acid-gated Cl- currents. Mol Pharmacol 39:691-696.
Ralvenius WT, Acuna MA, Benke D, Matthey A, Daali Y, Rudolph U, Desmeules J, Zeilhofer HU, Besson M (2016) The clobazam metabolite N-desmethyl clobazam is an alpha2 preferring benzodiazepine with an improved therapeutic window for antihyperalgesia. Neuropharmacology 109:366-375.
Ralvenius WT, Benke D, Acuna MA, Rudolph U, Zeilhofer HU (2015) Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABAA receptor subtype. Nat Commun 6:6803.
Ramerstorfer J, Furtmuller R, Vogel E, Huck S, Sieghart W (2010) The point mutation gamma 2F77I changes the potency and efficacy of benzodiazepine site ligands in different GABAA receptor subtypes. Eur J Pharmacol 636:18-27.
Randic M, Jiang MC, Cerne R (1993) Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. J Neurosci 13:5228-5241.
Reichling DB, Levine JD (2009) Critical role of nociceptor plasticity in chronic pain. Trends Neurosci 32:611-618.
Reinold H, Ahmadi S, Depner UB, Layh B, Heindl C, Hamza M, Pahl A, Brune K, Narumiya S, Muller U, Zeilhofer HU (2005) Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. J Clin Invest 115:673-679.
Rexed B (1952) The cytoarchitectonic organization of the spinal cord in the cat. J Comp Neurol 96:414-495.
Reynolds DV (1969) Surgery in the rat during electrical analgesia induced by focal brain stimulation. Science 164:444-445.
Rivas FM, Stables JP, Murphree L, Edwankar RV, Edwankar CR, Huang S, Jain HD, Zhou H, Majumder S, Sankar S, Roth BL, Ramerstorfer J, Furtmuller R, Sieghart W, Cook

JM (2009) Antiseizure activity of novel gamma-aminobutyric acid (A) receptor subtype-selective benzodiazepine analogues in mice and rat models. J Med Chem 52:1795-1798.
Roberts LA, Beyer C, Komisaruk BR (1986) Nociceptive responses to altered GABAergic activity at the spinal cord. Life Sci 39:1667-1674.
Rudolph U, Knoflach F (2011) Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. Nat Rev Drug Discov 10:685-697.
Rudolph U, Mohler H (2006) GABA-based therapeutic approaches: GABAA receptor subtype functions. Curr Opin Pharmacol 6:18-23.
Russell MG, Carling RW, Street LJ, Hallett DJ, Goodacre S, Mezzogori E, Reader M, Cook SM, Bromidge FA, Newman R, Smith AJ, Wafford KA, Marshall GR, Reynolds DS, Dias R, Ferris P, Stanley J, Lincoln R, Tye SJ, Sheppard WF, Sohal B, Pike A, Dominguez M, Atack JR, Castro JL (2006) Discovery of imidazo[1,2-b][1,2,4]triazines as GABA(A) alpha2/3 subtype selective agonists for the treatment of anxiety. J Med Chem 49:1235-1238.
Sandkuhler J (2009) Models and mechanisms of hyperalgesia and allodynia. Physiol Rev 89:707-758.
Sandkuhler J, Chen JG, Cheng G, Randic M (1997) Low-frequency stimulation of afferent Adelta-fibers induces long-term depression at primary afferent synapses with substantia gelatinosa neurons in the rat. J Neurosci 17:6483-6491.
Schaible HG, Richter F (2004) Pathophysiology of pain. Langenbecks Arch Surg 389:237-243.
Scholz J, Woolf CJ (2002) Can we conquer pain? Nat Neurosci 5 Suppl:1062-1067.
Scott-Stevens P, Atack JR, Sohal B, Worboys P (2005) Rodent pharmacokinetics and receptor occupancy of the GABAA receptor subtype selective benzodiazepine site ligand L-838417. Biopharm Drug Dispos 26:13-20.
Shalaly ND, Aneiros E, Blank M, Mueller J, Nyman E, Blind M, Dabrowski MA, Andersson CV, Sandberg K (2015) Positive Modulation of the Glycine Receptor by Means of Glycine Receptor-Binding Aptamers. J Biomol Screen 20:1112-1123.
Sieghart W (1995) Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. Pharmacol Rev 47:181-234.
Sigel E, Buhr A (1997) The benzodiazepine binding site of GABAA receptors. Trends Pharmacol Sci 18:425-429.
Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA (2004) Analysis of the set of GABA(A) receptor genes in the human genome. J Biol Chem 279:41422-41435.
Sivilotti L, Woolf CJ (1994) The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord. J Neurophysiol 72:169-179.
Smart TG, Moss SJ, Xie X, Huganir RL (1991) GABAA receptors are differentially sensitive to zinc: dependence on subunit composition. Br J Pharmacol 103:1837-1839.
Sonner JM, Zhang Y, Stabernack C, Abaigar W, Xing Y, Laster MJ (2003) GABA(A) receptor blockade antagonizes the immobilizing action of propofol but not ketamine or isoflurane in a dose-related manner. Anesth Analg 96:706-712, table of contents.
Stead C, Brown A, Adams C, Nickolls SJ, Young G, Kammonen J, Pryde D, Cawkill D (2016) Identification of Positive Allosteric Modulators of Glycine Receptors from a High-Throughput Screen Using a Fluorescent Membrane Potential Assay. J Biomol Screen.
Takahashi T, Momiyama A, Hirai K, Hishinuma F, Akagi H (1992) Functional correlation of fetal and adult forms of glycine receptors with developmental changes in inhibitory synaptic receptor channels. Neuron 9:1155-1161.
Talwar S, Lynch JW (2014) Phosphorylation mediated structural and functional changes in pentameric ligand-gated ion channels: implications for drug discovery. Int J Biochem Cell Biol 53:218-223.
Todd AJ (2010) Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci 11:823-836.

Todd AJ, Sullivan AC (1990) Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. J Comp Neurol 296:496-505.
Todd AJ, Watt C, Spike RC, Sieghart W (1996) Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord. J Neurosci 16:974-982.
Torebjork HE, Lundberg LE, LaMotte RH (1992) Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. J Physiol 448:765-780.
Tucker JC (1985) Benzodiazepines and the developing rat: a critical review. Neurosci Biobehav Rev 9:101-111.
Turecek R, Trussell LO (2001) Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. Nature 411:587-590.
Van Laere K, Bormans G, Sanabria-Bohorquez SM, de Groot T, Dupont P, De Lepeleire I, de Hoon J, Mortelmans L, Hargreaves RJ, Atack JR, Burns HD (2008) In vivo characterization and dynamic receptor occupancy imaging of TPA023B, an alpha 2/alpha 3/alpha 5 subtype selective gamma-aminobutyric acid-a partial agonist. Biol Psychiatry 64:153-161.
van Tulder MW, Touray T, Furlan AD, Solway S, Bouter LM (2003) Muscle relaxants for nonspecific low back pain: a systematic review within the framework of the cochrane collaboration. Spine (Phila Pa 1976) 28:1978-1992.
Vuilleumier PH, Besson M, Desmeules J, Arendt-Nielsen L, Curatolo M (2013) Evaluation of anti-hyperalgesic and analgesic effects of two benzodiazepines in human experimental pain: a randomized placebo-controlled study. PLoS One 8:e43896.
Wakita M, Kotani N, Akaike N (2016) Effects of propofol on glycinergic neurotransmission in a single spinal nerve synapse preparation. Brain Res 1631:147-156.
Waldvogel HJ, Baer K, Eady E, Allen KL, Gilbert RT, Mohler H, Rees MI, Nicholson LF, Faull RL (2010) Differential localization of gamma-aminobutyric acid type A and glycine receptor subunits and gephyrin in the human pons, medulla oblongata and uppermost cervical segment of the spinal cord: an immunohistochemical study. J Comp Neurol 518:305-328.
Waxman SG, Zamponi GW (2014) Regulating excitability of peripheral afferents: emerging ion channel targets. Nat Neurosci 17:153-163.
Wieland HA, Luddens H, Seeburg PH (1992) Molecular determinants in GABAA/BZ receptor subtypes. Adv Biochem Psychopharmacol 47:29-40.
Wisden W, Gundlach AL, Barnard EA, Seeburg PH, Hunt SP (1991) Distribution of GABAA receptor subunit mRNAs in rat lumbar spinal cord. Brain Res Mol Brain Res 10:179-183.
Witschi R, Johansson T, Morscher G, Scheurer L, Deschamps J, Zeilhofer HU (2010) Hoxb8-Cre mice: A tool for brain-sparing conditional gene deletion. Genesis 48:596-602.
Witschi R, Punnakkal P, Paul J, Walczak JS, Cervero F, Fritschy JM, Kuner R, Keist R, Rudolph U, Zeilhofer HU (2011) Presynaptic alpha2-GABAA receptors in primary afferent depolarization and spinal pain control. J Neurosci 31:8134-8142.
Woolf CJ (1996) Phenotypic modification of primary sensory neurons: the role of nerve growth factor in the production of persistent pain. Philos Trans R Soc Lond B Biol Sci 351:441-448.
Woolf CJ (2011) Central sensitization: implications for the diagnosis and treatment of pain. Pain 152:S2-15.
Wulff P, Goetz T, Leppa E, Linden AM, Renzi M, Swinny JD, Vekovischeva OY, Sieghart W, Somogyi P, Korpi ER, Farrant M, Wisden W (2007) From synapse to behavior: rapid modulation of defined neuronal types with engineered GABAA receptors. Nat Neurosci 10:923-929.
Xiong W, Cheng K, Cui T, Godlewski G, Rice KC, Xu Y, Zhang L (2011) Cannabinoid potentiation of glycine receptors contributes to cannabis-induced analgesia. Nat Chem Biol 7:296-303.

Xiong W, Cui T, Cheng K, Yang F, Chen SR, Willenbring D, Guan Y, Pan HL, Ren K, Xu Y, Zhang L (2012a) Cannabinoids suppress inflammatory and neuropathic pain by targeting alpha3 glycine receptors. J Exp Med 209:1121-1134.
Xiong W, Wu X, Li F, Cheng K, Rice KC, Lovinger DM, Zhang L (2012b) A common molecular basis for exogenous and endogenous cannabinoid potentiation of glycine receptors. J Neurosci 32:5200-5208.
Yaksh TL, Rudy TA (1978) Narcotic analgestics: CNS sites and mechanisms of action as revealed by intracerebral injection techniques. Pain 4:299-359.
Yamakura T, Bertaccini E, Trudell JR, Harris RA (2001) Anesthetics and ion channels: molecular models and sites of action. Annu Rev Pharmacol Toxicol 41:23-51.
Yevenes GE, Zeilhofer HU (2011a) Allosteric modulation of glycine receptors. Br J Pharmacol 164:224-236.
Yevenes GE, Zeilhofer HU (2011b) Molecular sites for the positive allosteric modulation of glycine receptors by endocannabinoids. PLoS One 6:e23886.
Yin H, Bhattarai JP, Oh SM, Park SJ, Ahn DK, Han SK (2016) Baicalin Activates Glycine and gamma-Aminobutyric Acid Receptors on Substantia Gelatinosa Neurons of the Trigeminal Subsnucleus Caudalis in Juvenile Mice. Am J Chin Med 44:389-400.
Zeilhofer HU, Benke D, Yevenes GE (2012) Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control. Annu Rev Pharmacol Toxicol 52:111-133.
Zeilhofer HU, Mohler H, Di Lio A (2009) GABAergic analgesia: new insights from mutant mice and subtype-selective agonists. Trends Pharmacol Sci 30:397-402.
Zeilhofer HU, Ralvenius WT, Acuna MA (2015) Restoring the spinal pain gate: GABA(A) receptors as targets for novel analgesics. Adv Pharmacol 73:71-96.
Zhang J, De Koninck Y (2006) Spatial and temporal relationship between monocyte chemoattractant protein-1 expression and spinal glial activation following peripheral nerve injury. J Neurochem 97:772-783.
Zhang Z, Cai YQ, Zou F, Bie B, Pan ZZ (2011) Epigenetic suppression of GAD65 expression mediates persistent pain. Nat Med 17:1448-1455.
Zhuo M (2011) Cortical plasticity as a new endpoint measurement for chronic pain. Mol Pain 7:54.

Curriculum Vitae

Title	BS, MS
First name	Mario Andrés
Last name	Acuña Miranda
Address	Altwiesenstrasse 197 8051 Zurich, Switzerland
Phone	+41 76 240 93 50
Email	mario.acuna@pharma.uzh.ch
Citizenship	Chile
Marital Status	Single
Date of Birth	19.12.1983

Professional Experience

Doctoral Research Fellow, **Institute of Pharmacology and Toxicology, University of Zurich**, Zurich, Switzerland — Feb 2012 – Present

Group of Prof. Dr. Hanns Ulrich Zeilhofer

Education

PhD Thesis at the Neuroscience Center Zurich (ZNZ) — Feb 2012 – Present
"Positive Allosteric Modulation of Inhibitory Neurotransmission in the spinal dorsal horn"
Institute of Pharmacology & Toxicology, University of Zurich, CH

Courses (PhD Studies) — 2012-2015

Postgraduate course in pharmacology and toxicology

ZNZ Neuroscience Introductory Course I & II — 2012/13

(Neurological Disorders, Neuroimmunology, Neuroimaging, Neuroanatomy, Synaptic Transmission, Neurodevelopment)

Crash Course in Statistics for Neuroscientists — 2013

Scientific writing for Neuroscientists — 2014

Master of Science in Neuroscience, Cellular And Molecular Biology (graduated with *Honors*) — Mar 2009 – Dec 2011
University of Chile, Chile

Bachelor of Science in Biology — Mar 2002 – Jul 2008
Catholic University of Valparaiso, Chile

Specialized Skills

Lab techniques

- specialization in electrophysiological techniques: whole-cell patch-clamp in cultured primary neurons, HEK239T cell line, and superficial spinal dorsal horn neurons
- HEK293T cell line culture
- transfection of HEK293T cell line
- immunohistochemistry techniques
- spinal cord slice preparation for electrophysiological analysis
- behavioural techniques in mice (routine chronic and acute pain tests, motor coordination and locomotor activity assays)

Computer skills

Wide experience in electrophysiological tools (HEKA Electronics®, Patch Master®, PClamp®, Mini Analysis®, IGOR®). Broad knowledge of Microsoft Office® and image processing software (Adobe Illustrator®, Adobe In Design®). Very good knowledge of Graph Pad Prism®. Basic knowledge of SPSS® and Matlab®.

Languages

Spanish	Mother tongue
English	Fully fluent, written and spoken
German	Good knowledge (Level B1.1)

Teaching Activities

Co-organizer and teacher in the yearly practical module "Ion Channels as Drug Targets" from the course "Methods in Experimental and Clinical Pharmacology – Module Bio405" at the Institute of Pharmacology and Toxicology, UZH, since 2012 to date.

Co-tutor of Master Thesis project: "Electrophysiological characterisation of dorsal horn gastrin-releasing peptide (GRP) neurons involved in spinal itch processing", 2015.

Publications

1. Effects of N-Glycosylation of the human cation channel TRPA1 on agonist-sensitivity. Biosci Rep., 2016. Egan TJ, ***Acuña MA,*** *Zenobi-Wong M, Zeilhofer HU, Urech D.*

2. The Clobazam Metabolite N-Desmethyl Clobazam is an α2 Preferring Benzodiazepine With an Improved Therapeutic Window for Antihyperalgesia. Neuropharm, 2016*. *Ralvenius WT, ****Acuña MA****, Benke D, Matthey A, Daali Y, Desmeules J, Rudolph U, Zeilhofer HU, Besson M*

3. Phosphorylation State-Dependent Modulation of Spinal Glycine Receptors Alleviates Inflammatory Pain. J Clin Invest, 2016. doi:10.1172/JCI83817. ***Acuña MA****, Yévenes GE, Ralvenius WT, Di Lio A, Lara CO, Muñoz B, Burgos CF, Moraga-Cid G, Corringer PJ, Zeilhofer HU.*

4. Concerted action of SUMOylation, acetylation and phosphorylation cascades regulate gephyrin scaffolding and GABAergic transmission. Nat Commun. 2016. *Ghosh H, Auguadri L, Battaglia S, Thirouin Z, Zemoura Z, Messner S,* ***Acuña MA****, Wildner H, Yévenes GE, Dieter A, Kawasaki H, Hottiger M, Zeilhofer HU, Fritschy JM, Tyagarajan SK.*

5. Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABAA receptor subtype. Nat Commun. 2015 Apr 13;6:6803. doi: 10.1038/ncomms7803. *Ralvenius WT, Benke D,* ***Acuña MA****, Rudolph U, Zeilhofer HU.*

6. Restoring the Spinal Pain Gate: GABAA Receptors as Targets for Novel Analgesics. Adv Pharmacol. Review. 2014. 2015;73:71-96. doi: 10.1016/bs.apha.2014.11.007. Epub 2015 Jan 14. *Zeilhofer HU, Ralvenius WT,* ***Acuña MA****.*

7. Ischemia-Like Oxygen and Glucose Deprivation Mediates Downregulation of Cell Surface GABAB via the ER Stress-Induced Transcription Factor CHOP. J Biol Chem. 2014 May 2; 214, 12896-12907. *Maier PJ, Zemoura K,* ***Acuña MA****, Yévenes GE, Zeilhofer HU, Benke D.*

8. Endoplasmic Reticulum Associated Protein Degradation Controls Cell Surface Expression of GABAB Receptors. J Biol Chem, 2013 Nov 29;288(48):34897-905. doi: 10.1074/jbc.M113.514745. Epub 2013 Oct 10. *Zemoura K, Schenkel M,* ***Acuña MA****, Yévenes GE, Zeilhofer HU and Benke D.*

9. Altered Voltage Dependent Calcium Currents in a Neuronal Cell Line Derived From the Cerebral Cortex of a Trisomy 16 Fetal Mouse, an Animal Model of Down Syndrome. Neurotox Res. 2012 Jul;22(1):59-68. doi: 10.1007/s12640-011-9304-5. Epub 2011 Dec 28. ***Acuña MA***, et al.

Acknowledgements

I express my sincere gratitude to my supervisor Prof. Dr. Hanns Ulrich Zeilhofer for his scientific guidance throughout my time in his group. I particularly consider all the time I spent in his office a valuable time of discussing science and learning how to see science with a critical eye. I deeply appreciate his trust on me, giving me several interesting projects which I always took as an opportunity to develop in science.

I would like to extend my gratitude to PD Dr. Dietmar Benke and Prof. Dr. Raimund Dutzler for being part of my PhD committee and for their important inputs. A special thank goes to Prof. Dr. Gonzalo E. Yévenes for his supervision and great disposition to everything (not only science-related) during his time as a postdoc in our group, and to Dr. William T. Ralvenius with whom I collaborated on several projects; to Dr. Khaled Zemoura, Dr. Patrick Maier and Dr. Shiva Tyagarajan and Dr. Tim Egan whom I collaborated with in several additional projects; to Thomas Grampp and Louis Scheurer for their amazing and professional technical assistance.

My most sincere appreciation goes to Dr. Robert Ganley and Ladina Hösli for their valuable support while writing this thesis.

Deep thank to the electrophysiology crew and all the (current and former) members of our group who created a great scientific atmosphere. I really appreciated all the moments we spent together, inside and outside the lab. And finally I want to thank all the members of the Institute of Pharmacology and Toxicology.